Excerpted *USP-NF* and *FCC* Standards: 
A COVID-19 Resource

A collection of standards provided as a resource to assist the healthcare industry with the challenges posed by COVID-19

*Not official text. Please refer to the currently official version of the applicable USP–NF or FCC standard for compliance purposes.*
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# GENERAL NOTICES AND REQUIREMENTS

Applying to Standards, Tests, Assays, and Other Specifications of the United States Pharmacopeia

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GENERAL NOTICES AND REQUIREMENTS

The General Notices and Requirements section (the General Notices) presents the basic assumptions, definitions, and default conditions for the interpretation and application of the United States Pharmacopeia (USP) and the National Formulary (NF).

Requirements stated in these General Notices apply to all articles recognized in the USP and NF (the “compendia”) and to all general chapters unless specifically stated otherwise.

Change to read:

1. TITLE AND REVISION

▲The full title of this joint compendium is The Pharmacopeia of the United States of America and the National Formulary Online (USP–NF Online). Although USP and NF are published together and share these General Notices, they are separate compendia. This is the 43rd revision of the USP and 38th edition of the NF. The final print publication of the USP–NF is USP 43–NF 38, after which official standards are published only in the USP–NF Online. Where the terms “USP,” “NF,” or “USP–NF” are used without further qualification, they refer to the currently official standard. ▲ (USP 1-Aug-2020)

Change to read:

2. OFFICIAL STATUS AND LEGAL RECOGNITION

2.10. Official Text

Official text of the USP and NF is published in the USP–NF Online (www.uspnf.com). ▲ (USP 1-Aug-2020)

Routine revisions are published in the USP–NF Online and become official on the date indicated, usually six months after publication.

▲Accelerated Revisions, published periodically in the USP–NF Online, are designed to make revisions official more quickly than through the routine process for publishing standards in the USP–NF Online. Accelerated Revisions may also be published on the Official Text section of USP’s website (https://www.uspnf.com/official-text). Accelerated Revisions supersede previously published content and become official on the date indicated.

Interim Revision Announcements are Accelerated Revisions to USP and NF that contain revisions and their official dates.

Revision Bulletins are Accelerated Revisions to official text or postponements that require expedited publication. They generally are official immediately unless otherwise specified in the Revision Bulletin.

Errata are Accelerated Revisions representing corrections to items erroneously published.

Periodically, a non-official volume of associated revisions and additions to the USP–NF is published in print and/or USB flash drive. This volume is intended to serve as a historical reference document and is not considered official text. ▲ (USP 1-Aug-2020)

2.20. Official Articles

An official article is an article that is recognized in USP or NF. An article is deemed to be recognized and included in a compendium when a monograph for the article is published in the compendium and an official date is generally or specifically assigned to the monograph.

The title specified in a monograph is the official title for such article. Other names considered to be synonyms of the official titles may not be used as substitutes for official titles. For drug products that incorporate a sensor to detect that the product has been administered, the official title shall be the title specified in the relevant drug product monograph plus the words “with sensor”.

Official articles include both official substances and official products. An official substance is a drug substance, excipient, dietary ingredient, other ingredient, or component of a finished device for which the monograph title includes no indication of the nature of the finished form.

An official product is a drug product, dietary supplement, compounded preparation, or finished device for which a monograph is provided.

2.30. Legal Recognition

The USP and NF are recognized in the laws and regulations of many countries throughout the world. Regulatory authorities may enforce the standards presented in the USP and NF, but because recognition of the USP and NF may vary by country, users should understand applicable laws and regulations. In the United States under the Federal Food, Drug, and Cosmetic Act (FDCA), both USP and NF are recognized as official compendia. A drug with a name recognized in USP–NF must comply with compendial identity standards or be deemed adulterated, misbranded, or both. See, e.g., FDCA § 501(b) and 502(e)(3)(b); also U.S. Food and Drug Administration (FDA) regulations, 21 CFR § 299.5(a&b). To avoid being deemed adulterated, such drugs must also comply with compendial standards for strength, quality, and purity, unless labeled to show all respects in which the drug differs. See, e.g., FDCA § 501(b) and 21 CFR § 299.5(c). In addition, to avoid being deemed misbranded, drugs recognized in USP–NF must also be packaged and labeled in compliance with compendial standards. See FDCA § 502(g).

A dietary supplement represented as conforming to specifications in USP will be deemed a misbranded food if it fails to so conform. See FDCA § 403(a)(2)(D).

Enforcement of USP standards is the responsibility of FDA and other government authorities in the U.S. and elsewhere. USP has no role in enforcement.

3. CONFORMANCE TO STANDARDS

3.10. Applicability of Standards

Standards for an article recognized in the compendia (USP–NF) are expressed in the article’s monograph, applicable general chapters, and General Notices. The identity, strength, quality, and purity of an article are determined by the official
tests, procedures, and acceptance criteria, and other requirements incorporated in the monograph, in applicable general chapters, or in the General Notices. “Applicable general chapters” means general chapters numbered below 1000 or above 2000 that are made applicable to an article through reference in General Notices, a monograph, or another applicable general chapter numbered below 1000. Where the requirements of a monograph differ from the requirements specified in these General Notices or an applicable general chapter, the monograph requirements apply and supersede the requirements of the General Notices or applicable general chapters, whether or not the monograph explicitly states the difference.

General chapters numbered 1000 to 1999 are for informational purposes only. They contain no mandatory tests, assays, or other requirements applicable to any official article, regardless of citation in a general chapter numbered below 1000, a monograph, or these General Notices. General chapters numbered above 2000 apply only to articles that are intended for use as dietary ingredients and dietary supplements. General chapter citations in NF monographs refer to USP general chapters.

Early adoption of revised standards in advance of the official date is allowed by USP unless specified otherwise at the time of publication. Where revised standards for an existing article have been published as final approved “official text” (as approved in section 2.10 Official Text) but have not yet reached the official date (6 months after publication, unless otherwise specified; see “official date”, section 2.20 Official Articles), compliance with the revised standard shall not preclude a finding or indication of conformance with compendial standards, unless USP specifies otherwise by prohibiting early adoption in a particular standard.

The standards in the relevant monograph, general chapter(s), and General Notices apply at all times in the life of the article from production to expiration. It is also noted that the manufacturer’s specifications, and manufacturing practices (e.g., Quality by Design, Process Analytical Technology, and Real Time Release Testing initiatives), generally are followed to ensure that the article will comply with compendial standards until its expiration date, when stored as directed. Every compendial article in commerce shall be so constituted that when examined in accordance with these assays and test procedures, it meets all applicable pharmacopeial requirements (General Notices, monographs, and general chapters). Thus, any official article is expected to meet the compendial standards if tested, and any official article actually tested as directed in the relevant monograph must meet such standards to demonstrate compliance.

Some tests, such as those for Dissolution and Uniformity of Dosage Units, require multiple dosage units in conjunction with a decision scheme. These tests, albeit using a number of dosage units, are in fact one determination. These procedures should not be confused with statistical sampling plans. The similarity to statistical procedures may seem to suggest an application to compendial testing, but such decisions are based on the reality of the testing. Frequency of testing and sampling are left to the preferences or direction of those performing compounding practice (whether by prescription, “over the counter,” or otherwise), as well as animal drugs. The applicable standard applies to such articles whether or not the added ingredient is an ingredient in a drug. Such articles (drug products, drug substances, excipients) include both human drugs (whether dispensed by prescription, “over the counter,” or otherwise), as well as animal drugs. The applicable standard applies to such articles whether or not the added designation “USP” or “NF” is used.

The applicable USP or NF standard applies to any article marketed in the United States that (1) is recognized in the compendium and (2) is intended or labeled for use as a drug or as an ingredient in a drug. Such articles (drug products, drug substances, excipients) include both human drugs (whether dispensed by prescription, “over the counter,” or otherwise), as well as animal drugs. The applicable standard applies to such articles whether or not the added designation “USP” or “NF” is used. The standards apply equally to articles bearing the official titles or names derived by transposition of the definitive words of official titles or transposition in the order of the names of two or more drug substances in official titles, or where there is use of synonyms with the intent or effect of suggesting a significant degree of identity with the official title or name.

3.10.20 Applicability of Standards to Medical Devices, Dietary Supplements, and Their Components and Ingredients

An article recognized in USP or NF shall comply with the compendial standards if the article is a medical device, component intended for a medical device, dietary supplement, dietary ingredient, or other ingredient that is intended for incorporation into a dietary supplement, and is labeled as conforming to the USP or NF. Generally, dietary supplements are prepared from ingredients that meet USP, NF, or Food Chemicals Codex standards. Where such standards do not exist, substances may be used in dietary supplements if they have been shown to be of acceptable food grade quality using other suitable procedures.

3.10.30 Applicability of Standards to the Practice of Compounding

USP compounding practice standards, Pharmaceutical Compounding—Nonsterile Preparations (795) and Pharmaceutical Compounding—Sterile Preparations (797), as applicable, apply to compounding practice or activity regardless of whether a monograph exists for the drug product or excipient. The compounding practice or activity is recognized in the compendium and (2) the article complies with the identity prescribed in the specified compendium.

When a drug product, drug substance, or excipient may use the designation “USP” or “NF” in conjunction with its official title or elsewhere on the label only when (1) a monograph is provided in the specified compendium and (2) the article complies with the identity prescribed in the specified compendium.
When a drug product, drug substance, compounded preparation, or excipient fails to comply with the identity prescribed in USP or NF or contains an added substance that interferes with the prescribed tests and procedures, the article shall be designated by a name that is clearly distinguishing and differentiating from any name recognized in USP or NF.

A medical device, dietary supplement, or ingredient or component of a medical device or dietary supplement may use the designation “USP” or “NF” in conjunction with its official title or elsewhere on the label only when (1) a monograph is provided in the specified compendium and (2) the article complies with the monograph standards and other applicable standards in that compendium.

The designation “USP” or “NF” on the label may not and does not constitute an endorsement by USP and does not represent assurance by USP that the article is known to comply with the relevant standards. USP may seek legal redress if an article purports to be or is represented as an official article in one of USP’s compendia and such claim is determined by USP not to be made in good faith.

The designation “USP–NF” may be used on the label of an article that provided the label also bears a statement such as “Meets NF standards as published by USP,” indicating the particular compendium to which the article purports to apply.

When the letters “USP,” “NF,” or “USP–NF” are used on the label of an article to indicate compliance with compendial standards, the letters shall appear in conjunction with the official title of the article. The letters are not to be enclosed in any symbol such as a circle, square, etc., and shall appear in capital letters.

If a dietary supplement does not comply with all applicable compendial requirements but contains one or more dietary ingredients or other ingredients that are recognized in USP or NF, the individual ingredient(s) may be designated as complying with USP or NF standards or being of USP or NF quality provided that the designation is limited to the individual ingredient(s) and does not suggest that the dietary supplement complies with USP standards.

4. MONOGRAPHS AND GENERAL CHAPITERS

4.10. Monographs

Monographs set forth the article’s name, definition, specification, and other requirements related to packaging, storage, and labeling. The specification consists of tests, procedures, and acceptance criteria that help ensure the identity, strength, quality, and purity of the article. For general requirements relating to specific monograph sections, see section 5. Monograph Components.

Because monographs may not provide standards for all relevant characteristics, some official substances may conform to the USP or NF standard but differ with regard to nonstandardized properties that are relevant to their use in specific preparations. To assure substitutability in such instances, users may wish to ascertain functional equivalence or determine such characteristics before use.

4.10.10. Applicability of Test Procedures

A single monograph may include more than one test, procedure, and/or acceptance criterion for the same attribute. Unless otherwise specified in the monograph, all tests are requirements. In some cases, monograph instructions allow the selection of tests that reflect attributes of different manufacturers’ articles, such as different polymorphic forms, impurities, hydrates, and dissolution. Monograph instructions indicate the tests, procedures, and/or acceptance criteria to be used and the required labeling.

The order in which the tests are listed in the monograph is based on the order in which they are approved by the relevant Expert Committee for inclusion in the monograph. Test 1 is not necessarily the test for the innovator or for the reference product. Depending on monograph instructions, a labeling statement is not typically required if Test 1 is used.

4.10.20. Acceptance Criteria

The acceptance criteria allow for analytical error, for unavoidable variations in manufacturing and compounding, and for deterioration to an extent considered acceptable under practical conditions. The existence of compendial acceptance criteria does not constitute a basis for a claim that an official substance that more nearly approaches 100% purity “exceeds” compendial quality. Similarly, the fact that an article has been prepared to tighter criteria than those specified in the monograph does not constitute a basis for a claim that the article “exceeds” the compendial requirements.

An official product shall be formulated with the intent to provide 100% of the quantity of each ingredient declared on the label. Where the minimum amount of a substance present in a dietary supplement is required by law to be higher than the lower acceptance criterion allowed for in the monograph, the upper acceptance criterion contained in the monograph may be increased by a corresponding amount.

The acceptance criteria specified in individual monographs and in the general chapters for compounded preparations are based on such attributes of quality as might be expected to characterize an article compounded from suitable bulk drug substances and ingredients, using the procedures provided or recognized principles of good compounding practice, as described in these compendia.

4.20. General Chapters

Each general chapter is assigned a number that appears in angle brackets adjacent to the chapter name (e.g., Chromatography (621)). General chapters may contain the following:

- Descriptions of tests and procedures for application through individual monographs,
- Descriptions and specifications of conditions and practices for pharmaceutical compounding,
- General information for the interpretation of the compendial requirements,
- Descriptions of general pharmaceutical storage, dispensing, and packaging practices, or
- General guidance to manufacturers of official substances or official products.

When a general chapter is referenced in a monograph, acceptance criteria may be presented after a colon.

Some chapters may serve as introductory overviews of a test or of analytical techniques. They may reference other general chapters that contain techniques, details of the procedures, and, at times, acceptance criteria.

Change to read:

5. MONOGRAPH COMPONENTS

5.10. Molecular Formula

The use of the molecular formula for the official substance(s) named in defining the required strength of a compendial article is intended to designate the chemical entity or entities, as given in the complete chemical name of the article, having absolute (100%) purity.

5.20. Added Substances

Added substances are presumed to be unsuitable for inclusion in an official article and therefore prohibited, if their presence impairs the bioavailability, therapeutic efficacy, or safety of the official article; or they interfere with the assays and tests prescribed for determining compliance with the
compendial standards (see section 3.20 Indicating Conformance). The air in a container of an official article may, where appropriate, be evacuated or be replaced by carbon dioxide, helium, argon, or nitrogen, or by a mixture of these gases. The use of such gas need not be declared in the labeling.

5.20.10. Added Substances in Official Substances

Official substances may contain only the specific added substances that are permitted by the individual monograph. Such added substances shall not exceed the quantity required for providing their intended effect. Where such addition is permitted, the label shall indicate the name(s) and amount(s) of any added substance(s).

5.20.20. Added Substances (Excipients and Ingredients) in Official Products

Suitable substances and excipients such as antimicrobial agents, pharmaceutical bases, carriers, coatings, flavors, preservatives, stabilizers, and vehicles may be added to an official product to enhance its stability, usefulness, or elegance, or to facilitate its preparation, unless otherwise specified in the individual monograph. Added substances and excipients employed solely to impart color may be incorporated into official products other than those intended for parenteral or ophthalmic use, in accordance with the regulations pertaining to the use of colors issued by the FDA, provided such added substances or excipients are otherwise appropriate in all respects. (See also Injections and Implanted Drugs Products (1), Product Quality Tests Common to Parenteral Dosage Forms, Specific Tests, Vehicles and added substances, Added substances.)

The proportions of the substances constituting the base in ointment and suppository products and preparations may be varied to maintain a suitable consistency under different climatic conditions, provided that the concentrations of drug substances are not varied and provided that the bioavailability, therapeutic efficacy, and safety of the preparation are not impaired.

5.20.20.1. In Compounded Preparations

Compounded preparations for which a complete composition is given shall contain only the ingredients named in the formulas unless specifically exempted herein or in the individual monograph. Deviation from the specified processes or methods of compounding, although not from the ingredients or proportions thereof, may occur provided that the finished preparation conforms to the relevant standards and to preparations produced by following the specified process.

Where a monograph for a compounded preparation calls for an ingredient in an amount expressed on the dried basis, the ingredient need not be dried before use if due allowance is made for the water or other volatile substances present in the quantity taken.

Specially denatured alcohol formulas are available for use in accordance with federal statutes and regulations of the Internal Revenue Service. A suitable formula of specially denatured alcohol may be substituted for Alcohol in the manufacture of official preparations intended for internal or topical use, provided that the denaturant is volatile and does not remain in the finished product. A preparation that is intended for topical application to the skin may contain specially denatured alcohol, provided that the denaturant is either a usual ingredient in the preparation or a permissible added substance; in either case the denaturant shall be identified on the label of the topical preparation. Where a process is given in the individual monograph, any preparation compounded using denatured alcohol shall be identical to that prepared by the monograph process.

5.20.20.2. In Dietary Supplements

Additional ingredients may be added to dietary supplement products provided that the additional ingredients (1) comply with applicable regulatory requirements, and (2) do not interfere with the assays and tests prescribed for determining compliance with compendial standards.

5.30. Description and Solubility

Only where a quantitative solubility test is given in a monograph and is designated as such is it a test for purity. A monograph may include information regarding the article’s description. Information about an article’s “description and solubility” also is provided in the reference table Description and Relative Solubility of USP and NF Articles. The reference table merely denotes the properties of articles that comply with monograph standards. The reference table is intended primarily for those who use, prepare, and dispense drugs and/or related articles. Although the information provided in monographs and the information in the reference table may indirectly assist in the preliminary evaluation of an article, it is not intended to serve as a standard or test for purity.

The approximate solubility of a compendial substance is indicated by one of the following descriptive terms:

<table>
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<th>Descriptive Term</th>
<th>Parts of Solvent Required for 1 Part of Solute</th>
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<tr>
<td>Very soluble</td>
<td>Less than 1</td>
</tr>
<tr>
<td>Freely soluble</td>
<td>From 1 to 10</td>
</tr>
<tr>
<td>Soluble</td>
<td>From 10 to 30</td>
</tr>
<tr>
<td>Sparingly soluble</td>
<td>From 30 to 100</td>
</tr>
<tr>
<td>Slightly soluble</td>
<td>From 100 to 1,000</td>
</tr>
<tr>
<td>Very slightly soluble</td>
<td>From 1,000 to 10,000</td>
</tr>
<tr>
<td>Practically insoluble, or Insoluble</td>
<td>Greater than or equal to 10,000</td>
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5.40. Identification

A compendial test titled Identification is provided as an aid in verifying the identity of articles as they are purported to be, e.g., those taken from labeled containers, and to establish whether it is the article named in USP–NF. The Identification test for a particular article may consist of one or more procedures. When a compendial Identification test is undertaken, all requirements of all specified procedures in the test must be met to satisfy the requirements of the test. Failure of an article to meet all the requirements of a prescribed Identification test (i.e., failure to meet the requirements of all of the specified procedures that are components of that test) indicates that the article is mislabeled and/or adulterated.

5.50. Assay

Assay tests for compounded preparations are not intended for evaluating a compounded preparation before dispensing, but instead are intended to serve as the official test in the event of a question or dispute regarding the preparation’s conformance to official standards.

5.50.10. Units of Potency (Biological)

For substances that cannot be completely characterized by chemical or physical means or that need confirmation of functionality or tertiary structure, it may be necessary to express quantities of biological activity in units of biological potency, each defined by an authoritative, designated reference standard. In cases where international reference materials have been discontinued, international units of potency may be defined in terms of molecular mass, such as in the cases of vitamins A, D, and E.

Where available, World Health Organization (WHO) international biological standards define the International Biological Reference Preparation (IBRP) and the International Reference Preparation (IRP).
Units (IU). USP monographs refer to the units assigned by USP Reference Standards either directly as International Units (IU) or as “USP Units.” For some biological products, units of potency are value assigned against a corresponding U.S. Standard established by FDA, whether or not International Units or USP Units have been defined (see Biologics (1041)). Note that product-related labeling, e.g., on containers, need not use the full phrase “USP [product name] Units” that appears in many USP monograph labeling sections. The term “USP Units” can be used on product labeling consistent with USP compendial requirements, provided it is clear from the context that the potency is stated in terms of USP [product name] Units. In such circumstances it should be clear that “USP Units” and “USP [product name] Units” share the same meaning.

5.60. Impurities and Foreign Substances

Tests for the presence of impurities and foreign substances are provided to limit such substances to amounts that are unobjectionable under conditions in which the article is customarily employed (see also Impurities in Drug Substances and Drug Products (1086)).

Monomograph tests and acceptance criteria suitable for detecting and controlling impurities that may result from a change in the processing methods or that may be introduced from external sources should be employed in addition to the tests provided in the individual monograph, where the presence of the impurity is inconsistent with applicable good manufacturing practices or good pharmaceutical practices.

5.60.10. Other Impurities in USP and NF Articles

If a USP or NF monograph includes an assay or organic impurity test based on chromatography, other than a test for residual solvents, and that monograph procedure does not detect an impurity present in the substance, the amount and identity of the impurity, where both are known, shall be stated in the labeling (certificate of analysis) of the official substance, under the heading Other Impurity(ies).

The presence of any unlabeled other impurity in an official substance is a variance from the standard if the content is 0.1% or greater. The sum of all Other Impurities combined with the monograph-detected impurities may not exceed 2.0% (see Ordinary Impurities (466)), unless otherwise stated in the monograph.

The following categories of drug substances are excluded from Other Impurities requirements:

- Fermentation products and semi-synthetics derived therefrom,
- Radiopharmaceuticals,
- Biologics,
- Biotechnology-derived products,
- Peptides,
- Herbals, and
- Crude products of animal or plant origin.

Any substance known to be toxic shall not be listed under Other Impurities.

5.60.20. Residual Solvents in USP and NF Articles

All USP and NF articles are subject to relevant control of residual solvents, even when no test is specified in the individual monograph. If solvents are used during production, they must be of suitable quality. In addition, the toxicity and residual level of each solvent shall be taken into consideration, and the solvents limited according to the principles defined and the requirements specified in Residual Solvents (467), using the general methods presented therein or other suitable methods.

5.60.30. Elemental Impurities in USP Drug Products and Dietary Supplements

Elemental impurities in official drug products are controlled according to the principles defined and requirements specified in Elemental Impurities—Limits (232). Elemental contaminants in official dietary supplements are controlled according to the principles defined and requirements specified in Elemental Contaminants in Dietary Supplements (2232).

5.70. Performance Tests

Where content uniformity determinations have been made using the same analytical methodology specified in the Assay, with appropriate allowances made for differences in sample preparation, the average of all of the individual content uniformity determinations may be used as the Assay value.

5.80. USP Reference Standards

USP Reference Standards are authentic specimens that have been approved as suitable for use ▲ (USP 1-Aug-2020 in USP or NF tests and assays (see USP Reference Standards (11)). Where USP or NF tests or assays call for the use of a USP Reference Standard, only those results obtained using the specified USP Reference Standard are conclusive. Where a procedure calls for the use of a compendial article rather than for a USP Reference Standard as a material standard of reference, a substance meeting all of the compendial monograph requirements for that article shall be used. If any new USP or NF standard requires the use of a new USP Reference Standard that is not yet available, that portion of the standard containing the requirement shall not be official until the specified USP reference material is available.

Announcements of the availability of new USP Reference Standards and announcements of tests or procedures that are held in abeyance pending availability of required USP Reference Standards are also available on the “Official Text” tab of USP’s website (www.uspnf.com). ▲ (USP 1-Aug-2020) Unless otherwise directed in the procedure in the individual monograph or in a general chapter, USP Reference Standards are to be used in accordance with the instructions on the label of the Reference Standard.

6. TESTING PRACTICES AND PROCEDURES

6.10. Safe Laboratory Practices

In performing compendial procedures, safe laboratory practices shall be followed, including precautionary measures, protective equipment, and work practices consistent with the chemicals and procedures used. Before undertaking any procedure described in the compendia, the analyst should be aware of the hazards associated with the chemicals and the techniques and means of protecting against them. These compendia are not designed to describe such hazards or protective measures.

6.20. Automated Procedures

Automated and manual procedures employing the same basic chemistry are considered equivalent provided the automated system is properly qualified as being suitable to execute the compendial manual method and the analytical procedure is verified under the new equipment conditions.

6.30. Alternative and Harmonized Methods and Procedures

An alternative method or procedure is defined as any method or procedure other than the compendial method or procedure for the article in question. The alternative method or procedure must be fully validated (see Validation of Compendial Procedures (1225)) and must produce comparable results to the compendial method or procedure within allowable limits established on a case-by-case basis. Alternative methods or procedures can be developed for any one of a number of reasons not limited to simplification of sample preparation, enhanced precision and accuracy, improved (shortened) run time, or being better suited to automation than the compendial method or procedure. Only those results
obtained by the methods and procedures given in the compendia are conclusive.

For evaluation as a potential replacement or addition to the standard, alternative methods and procedures should be submitted to USP (see section 4.10 Monographs).

Certain general chapters contain a statement that the text in question is harmonized with the corresponding text of the European Pharmacopoeia and/or the Japanese Pharmacopoeia and that these texts are interchangeable. Therefore, if a substance or preparation is found to comply with a requirement using an interchangeable method or procedure from one of these pharmacopoeias, it should comply with the requirements of the USP–NF. When a difference appears, or in the event of dispute, only the result obtained by the method and/or procedure given in the USP–NF is conclusive.

6.40. Dried, Anhydrous, Ignited, or Solvent-Free Basis

All calculations in the compendia assume an “as-is” basis unless otherwise specified.

Test procedures may be performed on the undried or unignited substance and the results calculated on the dried, anhydrous, or ignited basis, provided a test for Loss on Drying, or Water Determination, or Loss on Ignition, respectively, is given in the monograph. Where the presence of moisture or other volatile material may interfere with the procedure, previous drying of the substance is specified in the individual monograph and is obligatory.

The term “solvent-free” signifies that the calculation shall be corrected for the presence of known solvents as determined using the methods described in (467) unless a test for limit of organic solvents is provided in the monograph.

The term “previously dried” without qualification signifies that the substance shall be dried as directed under Loss on Drying (731) or Water Determination (921) (gravimetric determination).

Where drying in vacuum over a desiccant is directed, a vacuum desiccator, a vacuum drying pistol, or other suitable vacuum drying apparatus shall be used.

6.40.10. Ignite to Constant Weight

“Ignite to constant weight” means that ignition shall be continued at 800 ± 25°, unless otherwise indicated, until two consecutive weighings, the second of which is taken after an additional drying period appropriate to the nature and quantity of the residue, do not differ by more than 0.50 mg per g of substance taken.

6.40.20. Dried to Constant Weight

“Dried to constant weight” means that drying shall be continued until two consecutive weighings, the second of which is taken after an additional drying period appropriate to the nature and quantity of the residue, do not differ by more than 0.50 mg per g of substance taken.

6.50. Preparation of Solutions

6.50.10. Filtration

Where a procedure gives direction to “filter” without further qualification, the liquid shall be passed through suitable filter paper or equivalent device until the filtrate is clear. Due to the possibility of filter effects, the initial volumes of a filtrate may be discarded.

6.50.20. Solutions

Unless otherwise specified, all solutions shall be prepared with Purified Water. Solutions for quantitative measures shall be prepared using accurately weighed or accurately measured analytes (see section 8.20 About).

An expression such as “(20:5:2)” means that the respective numbers of parts, by volume, of the designated liquids shall be mixed, unless otherwise indicated.

6.50.20.1. Adjustments to Solutions

When a specified concentration is called for in a procedure, a solution of other normality or molarity may be used, provided that allowance is made for the difference in concentration and that the change does not increase the error of measurement.

Proportionately larger or smaller quantities than the specified weights and volumes of assay or test substances and Reference Standards may be taken, provided the measurement is made with at least equivalent accuracy.

Unless otherwise indicated, analyte concentrations shall be prepared to within ten percent (10%) of the indicated value. In the case in which a procedure is adapted to the working range of an instrument, solution concentrations may differ from the indicated value by more than ten percent (10%), with appropriate changes in associated calculations. Any changes shall fall within the validated range of the instrument.

When adjustment of pH is indicated with either an acid or base and the concentration is not indicated, appropriate concentrations of that acid or base may be used.

6.50.20.2. Test Solutions

Information on Test Solutions (TS) is provided in the Test Solutions portion of the Reagents, Indicators, and Solutions section of the USP–NF. Use of an alternative Test Solution or a change in the Test Solution used may require validation.

6.50.3. Indicator Solutions

Where a procedure specifies the use of an indicator TS, approximately 0.2 mL, or 3 drops, of the solution shall be added unless otherwise directed.

6.60. Units Necessary to Complete a Test

Unless otherwise specified, a sufficient number of units to ensure a suitable analytical result shall be taken.

6.60.10. Tablets

Where the procedure of a Tablet monograph directs to weigh and finely powder not fewer than a given number of Tablets, a counted number of Tablets shall be weighed and reduced to a powder. The portion of the powdered Tablets taken shall be representative of the whole Tablets and shall, in turn, be weighed accurately.

6.60.20. Capsules

Where the procedure of a Capsule monograph gives direction to remove, as completely as possible, the contents of not fewer than a given number of the Capsules, a counted number of Capsules shall be carefully opened and the contents quantitatively removed, combined, mixed, and weighed accurately. The portion of mixed Capsules contents taken shall be representative of the contents of the Capsules and shall, in turn, be weighed accurately.

6.70. Reagents

The proper conduct of the compendial procedures and the reliability of the results depend, in part, upon the quality of the reagents used in the performance of the procedures. Unless otherwise specified, reagents conforming to the specifications set forth in the current edition of Reagent Chemicals published by the American Chemical Society (ACS) shall be used. Where such ACS reagent specifications are not available or where the required purity differs, compendial specifications for reagents of acceptable quality are provided (see the Reagents, Indicators, and Solutions section of the USP–NF). Reagents not covered by any of these specifications should be of a grade suitable to the proper performance of the method of assay or test involved.

Listing of these reagents, including the indicators and solutions employed as reagents, in no way implies that they have therapeutic utility; furthermore, any reference to USP or
Not official text. Please refer to the currently official version of the applicable USP–NF or FCC standard for compliance purposes.

NF in their labeling shall include also the term “reagent” or “reagent grade.” USP may supply reagents if they otherwise may not be generally commercially available.

6.80. Equipment

Unless otherwise specified, a specification for a definite size or type of container or apparatus in a procedure is given solely as a recommendation. Other dimensions or types may be used if they are suitable for the intended use.

6.80.10. Apparatus for Measurement

Where volumetric flasks or other exact measuring, weighing, or sorting devices are specified, this or other equipment of at least equivalent accuracy shall be employed.

6.80.10.1. Pipet/Pipette

Where a pipet/pipette is specified, a suitable buret may be substituted. Where a “to contain” pipet/pipette is specified, a suitable volumetric flask may be substituted.

6.80.10.2. Light Protection

Where low-actinic or light-resistant containers are specified, either containers specially treated to protect contents from light or clear containers that have been rendered opaque by application of a suitable coating or wrapping may be used.

6.80.20. Instrumental Apparatus

An instrument may be substituted for the specified instrument if the substitute uses the same fundamental principles of operation and is of equivalent or greater sensitivity and accuracy. These characteristics shall be qualified as appropriate. Where a particular brand or source of a material, instrument, or piece of equipment, or the name and address of a manufacturer or distributor, is mentioned (ordinarily in a footnote), this identification is furnished solely for informational purposes as a matter of convenience, without implication of approval, endorsement, or certification.

6.80.20.1. Chromatographic Tubes and Columns

The term “diameter” refers to internal diameter (ID).

6.80.20.2. Tubing

The term “diameter” refers to outside diameter (OD).

6.80.20.3. Steam Bath

Where use of a steam bath is directed, use actively flowing steam or another regulated heat source controlled at an equivalent temperature.

6.80.20.4. Water Bath

A water bath requires vigorously boiling water unless otherwise specified.

6.80.30. Temperature Reading Devices

Temperature reading devices suitable for pharmacopeial tests conform to specifications that are traceable to a National Institute of Standards and Technology (NIST) standard or equivalent. Temperature reading devices may be of the liquid-in-glass type or an analog or digital temperature indicator type, such as a resistance temperature device, thermistor, or thermocouple. Standardization of thermometers is performed on an established testing frequency with a temperature standard traceable to NIST. For example, refer to the current issue of American Society of Testing and Materials (ASTM) standards E1 for liquid-in-glass thermometers.

7. TEST RESULTS

7.10. Interpretation of Requirements

Analytical results observed in the laboratory (or calculated from experimental measurements) are compared with stated acceptance criteria to determine whether the article conforms to compendial requirements.

The reportable value, which often is a summary value for several individual determinations, is compared with the acceptance criteria. The reportable value is the end result of a completed measurement procedure, as documented.

Where acceptance criteria are expressed numerically herein through specification of an upper and/or lower limit, permitted values include the specified values themselves, but no values outside the limit(s). Acceptance criteria are considered significant to the last digit shown.

7.10.5. Nominal Concentrations in Equations

Where a “nominal concentration” is specified, calculate the concentration based on the label claim. In assay procedures, water correction is typically stated in the Definition and on the label of the USP Reference Standard. For other procedures, correction for assayed content, potency, or both is made prior to using the concentration in the equation provided in the monograph.

7.10.10. Equivalence Statements in Titrimetric Procedures

The directions for titrimetric procedures conclude with a statement of the weight of the analyte that is equivalent to each mL of the standardized titrant. In such an equivalence statement, the number of significant figures in the concentration of the titrant should be understood to correspond to the number of significant figures in the weight of the analyte. Corrections to calculations based on the blank determination are to be made for all titrimetric assays where appropriate (see Titrimetry (541)).

7.20. Rounding Rules

The observed or calculated values shall be rounded off to the number of decimal places that is in agreement with the limit expression. Numbers should not be rounded until the final calculations for the reportable value have been completed. Intermediate calculations (e.g., slope for linearity) may be rounded for reporting purposes, but the original (not rounded) value should be used for any additional required calculations. Acceptance criteria are fixed numbers and are not rounded.

When rounding is required, consider only one digit in the decimal place to the right of the last place in the limit expression. If this digit is smaller than 5, it is eliminated and the preceding digit is unchanged. If this digit is equal to or greater than 5, it is eliminated and the preceding digit is increased by 1.

8. TERMS AND DEFINITIONS

8.10. Abbreviations

- RS refers to a USP Reference Standard.
- CS refers to a Colorimetric Solution.
- TS refers to a Test Solution.
- VS refers to a Volumetric Solution that is standardized in accordance with directions given in the individual monograph or in the Reagents, Indicators, and Solutions section of USP–NF.

8.20. About

“About” indicates a quantity within 10%.

If the measurement is stated to be “accurately measured” or “accurately weighed,” follow the statements in Volumetric Apparatus (31) and Balances (41), respectively.

8.30. Alcohol Content

Percentages of alcohol, such as those under the heading Alcohol Content, refer to percentage by volume of C<sub>2</sub>H<sub>5</sub>OH at 15.56°. Where a formula, test, or assay calls for alcohol, ethyl alcohol, or ethanol, the USP monograph article Alcohol shall be used. Where reference is made to “C<sub>2</sub>H<sub>5</sub>OH,” absolute (100%) ethanol is intended. Where a procedure calls for dehydrated alcohol, alcohol absolute, or anhydrous alcohol, the USP monograph article Dehydrated Alcohol shall be used.

8.40. Atomic Weights

Atomic weights used in computing molecular weights and the factors in the assays and elsewhere are those established...
by the IUPAC Commission on Isotopic Abundances and Atomic Weights.

8.50. Blank Determinations
Where it is directed that “any necessary correction” be made by a blank determination, the determination shall be conducted using the same quantities of the same reagents treated in the same manner as the solution or mixture containing the portion of the substance under assay or test, but with the substance itself omitted.

8.60. Concomitantly
“Concomitantly” denotes that the determinations or measurements are to be performed in immediate succession.

8.70. Desiccator
The instruction “in a desiccator” indicates use of a tightly closed container of suitable size and design that maintains an atmosphere of low moisture content by means of a suitable desiccant such as anhydrous calcium chloride, magnesium perchlorate, phosphorus pentoxide, or silica gel. See also section 8.220 Vacuum Desiccator.

8.80. Logarithms
Logarithms are to the base 10.

8.90. Microbial Strain
A microbial strain cited and identified by its American Type Culture Collection (ATCC) catalog number shall be used directly or, if subcultured, shall be used not more than five passages removed from the original strain.

8.100. Negligible
“Negligible” indicates a quantity not exceeding 0.50 mg.

8.110. NLT/NMT
“NLT” means “not less than.” “NMT” means “not more than.”

8.120. Odor
“Odorless,” “practically odorless,” “a faint characteristic odor,” and variations thereof indicate evaluation of a suitable quantity of freshly opened material after exposure to the air for 15 minutes. An odor designation is descriptive only and should not be regarded as a standard of purity for a particular lot of an article.

8.130. Percent
“Percent” used without qualification means:
- For mixtures of solids and semisolids, percent weight in weight;
- For solutions or suspensions of solids in liquids, percent weight in volume;
- For solutions of liquids in liquids, percent volume in volume;
- For solutions of gases in liquids, percent weight in volume.

For example, a 1 percent solution is prepared by dissolving 1 g of a solid or semisolid, or 1 mL of a liquid, in sufficient solvent to make 100 mL of the solution.

8.140. Percentage Concentrations
Percentage concentrations are expressed as follows:
- Percent Weight in Weight (w/w) is defined as the number of g of a solute in 100 g of solution.
- Percent Weight in Volume (w/v) is defined as the number of g of a solute in 100 mL of solution.
- Percent Volume in Volume (v/v) is defined as the number of mL of a solute in 100 mL of solution.

8.150. Pressure
Pressure is determined by use of a suitable manometer or barometer calibrated in terms of the pressure exerted by a column of mercury of the stated height.

8.160. Reaction Time
Reaction time is 5 minutes unless otherwise specified.

8.170. Specific Gravity
Specific gravity is the weight of a substance in air at 25° divided by the weight of an equal volume of water at the same temperature.

8.180. Temperatures
Temperatures are expressed in centigrade (Celsius) degrees, and all measurements are made at 25° unless otherwise indicated. Where moderate heat is specified, any temperature not higher than 45° (113° F) is indicated.

8.190. Time
Unless otherwise specified, rounding rules, as described in section 7.20 Rounding Rules, apply to any time specified.

8.200. Transfer
“Transfer” indicates a quantitative manipulation.

“Vacuum” denotes exposure to a pressure of less than 20 mm of mercury (2.67 kPas), unless otherwise indicated.

8.220. Vacuum Desiccator
“Vacuum desiccator” indicates a desiccator that maintains a low-moisture atmosphere at a reduced pressure of not more than 20 mm of mercury (2.67 kPas) or at the pressure designated in the individual monograph.

Illustration of Rounding Numerical Values for Comparison with Requirements

<table>
<thead>
<tr>
<th>Compendial Requirement</th>
<th>Unrounded Value</th>
<th>Rounded Result</th>
<th>Conforms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assay limit ≥98.0%</td>
<td>97.96%</td>
<td>98.0%</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>97.92%</td>
<td>97.9%</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>97.95%</td>
<td>98.0%</td>
<td>Yes</td>
</tr>
<tr>
<td>Assay limit ≤101.5%</td>
<td>101.55%</td>
<td>101.6%</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>101.46%</td>
<td>101.5%</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>101.45%</td>
<td>101.5%</td>
<td>Yes</td>
</tr>
<tr>
<td>Limit test ≤0.02%</td>
<td>0.025%</td>
<td>0.03%</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>0.015%</td>
<td>0.02%</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>0.027%</td>
<td>0.03%</td>
<td>No</td>
</tr>
<tr>
<td>Limit test ≤3 ppm</td>
<td>3.5 ppm</td>
<td>4 ppm</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>3.4 ppm</td>
<td>3 ppm</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>2.5 ppm</td>
<td>3 ppm</td>
<td>Yes</td>
</tr>
</tbody>
</table>
8.230. Water
8.230.10. Water as an Ingredient in an Official Product
As an ingredient in an official product, water meets the requirements of the appropriate water monograph in USP or NF.

When used in the manufacture of official substances, water shall meet the requirements for drinking water as set forth in the U.S. Environmental Protection Agency National Primary Drinking Water Regulations or in the drinking water regulations of the European Union or of Japan, or in the World Health Organization’s Guidelines for Drinking Water Quality. Additional specifications may be required in monographs.

8.230.30. Water in a Compendial Procedure
When water is called for in a compendial procedure, the USP monograph article Purified Water shall be used unless otherwise specified. Definitions for other types of water are provided in Reagents, Indicators, and Solutions and in Water for Pharmaceutical Purposes (1231).

8.240. Weights and Measures
In general, weights and measures are expressed in the International System of Units (SI) as established and revised by the Conférence générale des poids et mesures. For compendial purposes, the term “weight” is considered to be synonymous with “mass.”

Molality is designated by the symbol $m$ preceded by a number that represents the number of moles of the designated solute contained in 1 kilogram of the designated solvent.

Molarity is designated by the symbol $M$ preceded by a number that represents the number of moles of the designated solute contained in an amount of the designated solvent that is sufficient to prepare 1 liter of solution.

Normality is designated by the symbol $N$ preceded by a number that represents the number of equivalents of the designated solute contained in an amount of the designated solvent that is sufficient to prepare 1 liter of solution.

The symbol for degrees (°) without a qualifying unit of measure represents degrees Celsius.

Chart of Symbols and Prefixes commonly employed for SI metric units and other units:

<table>
<thead>
<tr>
<th>Units</th>
<th>Symbol</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length</td>
<td></td>
<td></td>
</tr>
<tr>
<td>meter</td>
<td>m</td>
<td></td>
</tr>
<tr>
<td>centimeter</td>
<td>cm</td>
<td></td>
</tr>
<tr>
<td>millimeter</td>
<td>mm</td>
<td></td>
</tr>
<tr>
<td>micrometer</td>
<td>µm</td>
<td>Previously referred to as a micron</td>
</tr>
<tr>
<td>nanometer</td>
<td>nm</td>
<td>Previously the symbol μm (for millimicron) was used</td>
</tr>
<tr>
<td>Ångström</td>
<td>Å</td>
<td>Equal to 0.1 nm</td>
</tr>
<tr>
<td>Mass</td>
<td></td>
<td></td>
</tr>
<tr>
<td>kilogram</td>
<td>kg</td>
<td></td>
</tr>
<tr>
<td>gram</td>
<td>g</td>
<td></td>
</tr>
<tr>
<td>milligram</td>
<td>mg</td>
<td></td>
</tr>
<tr>
<td>Amount of Substance</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mole</td>
<td>mol</td>
<td>Historically referred to as gram-molecular weight or gram-atomic weight</td>
</tr>
<tr>
<td>millimole</td>
<td>mmol</td>
<td></td>
</tr>
<tr>
<td>micromole</td>
<td>µmol</td>
<td></td>
</tr>
<tr>
<td>femt mole</td>
<td>fmol</td>
<td></td>
</tr>
<tr>
<td>equivalent</td>
<td>Eq</td>
<td>Also referred to as gram-equivalent weight. It is used in the calculation of substance concentration in units of normality. This unit is no longer preferred for use in analytical chemistry or metrology.</td>
</tr>
<tr>
<td>mili equivalent</td>
<td>mEq</td>
<td></td>
</tr>
<tr>
<td>os mole</td>
<td>Osmol</td>
<td>Osmotic pressure of a solution, related to substance concentration.</td>
</tr>
<tr>
<td>millios mole</td>
<td>mOs mol</td>
<td></td>
</tr>
<tr>
<td>Pressure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pascal</td>
<td>Pa</td>
<td></td>
</tr>
<tr>
<td>kilopascal</td>
<td>kPa</td>
<td></td>
</tr>
<tr>
<td>pounds per square inch</td>
<td>psi</td>
<td></td>
</tr>
<tr>
<td>millimeter of mercury</td>
<td>mmHg</td>
<td>Equal to 133.322 Pa</td>
</tr>
</tbody>
</table>
9. PRESCRIBING AND DISPENSING

9.10. Use of Metric Units
Prescriptions for compendial articles shall be written to state the quantity and/or strength desired in metric units unless otherwise indicated in the individual monograph [see also section 5.50.10 Units of Potency (Biological) above]. If an amount is prescribed by any other system of measurement, only an amount that is the metric equivalent of the prescribed amount shall be dispensed. Abbreviations for the terms “Units” or “International Units” shall not be used for labeling or prescribing purposes. Apothecary unit designations on labels and labeling shall not be used.

9.20. Changes in Volume
In the dispensing of prescription medications, slight changes in volume owing to variations in room temperatures may be disregarded.

10. PRESERVATION, PACKAGING, STORAGE, AND LABELING

10.10. Packaging and Storage
All articles in USP or NF are subject to the packaging and storage requirements specified in Packaging and Storage Requirements (659), unless different requirements are provided in an individual monograph.

10.20. Labeling
All articles in USP or NF are subject to the labeling requirements specified in Labeling (7), unless different requirements are provided in an individual monograph.

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### Electrical units

<table>
<thead>
<tr>
<th>Units</th>
<th>Symbol</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>ampere</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>volt</td>
<td>V</td>
<td></td>
</tr>
<tr>
<td>millivolt</td>
<td>mV</td>
<td>Unit of frequency</td>
</tr>
<tr>
<td>hertz</td>
<td>Hz</td>
<td></td>
</tr>
<tr>
<td>kilohertz</td>
<td>kHz</td>
<td></td>
</tr>
<tr>
<td>megahertz</td>
<td>MHz</td>
<td></td>
</tr>
<tr>
<td>electron volt</td>
<td>eV</td>
<td></td>
</tr>
<tr>
<td>kilo-electron volt</td>
<td>keV</td>
<td></td>
</tr>
<tr>
<td>mega-electron volt</td>
<td>MeV</td>
<td></td>
</tr>
</tbody>
</table>

### Radiation

<table>
<thead>
<tr>
<th>Units</th>
<th>Symbol</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>becquerel</td>
<td>Bq</td>
<td>SI unit of activity for radionuclides</td>
</tr>
<tr>
<td>kilobecquerel</td>
<td>kBq</td>
<td></td>
</tr>
<tr>
<td>megabecquerel</td>
<td>MBq</td>
<td></td>
</tr>
<tr>
<td>gigabecquerel</td>
<td>GBq</td>
<td></td>
</tr>
<tr>
<td>curie</td>
<td>Ci</td>
<td>Non-SI unit of activity for radionuclides</td>
</tr>
<tr>
<td>millicurie</td>
<td>mCi</td>
<td></td>
</tr>
<tr>
<td>microcurie</td>
<td>µCi</td>
<td></td>
</tr>
<tr>
<td>nanocurie</td>
<td>nCi</td>
<td></td>
</tr>
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</table>

### Other

<table>
<thead>
<tr>
<th>Units</th>
<th>Symbol</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>acceleration due to gravity</td>
<td>g</td>
<td>Used to express rate of centrifugation</td>
</tr>
<tr>
<td>revolutions per minute</td>
<td>rpm</td>
<td>Used to express rate of centrifugation</td>
</tr>
</tbody>
</table>

---

### Selected SI Prefixes

<table>
<thead>
<tr>
<th>Name</th>
<th>Symbol</th>
<th>Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>mega</td>
<td>M</td>
<td>$10^6$</td>
</tr>
<tr>
<td>kilo</td>
<td>k</td>
<td>$10^3$</td>
</tr>
<tr>
<td>deci</td>
<td>d</td>
<td>$10^{-1}$</td>
</tr>
<tr>
<td>centi</td>
<td>c</td>
<td>$10^{-2}$</td>
</tr>
<tr>
<td>milli</td>
<td>m</td>
<td>$10^{-3}$</td>
</tr>
<tr>
<td>micro</td>
<td>µ</td>
<td>$10^{-6}$</td>
</tr>
<tr>
<td>nano</td>
<td>n</td>
<td>$10^{-9}$</td>
</tr>
<tr>
<td>pico</td>
<td>p</td>
<td>$10^{-12}$</td>
</tr>
<tr>
<td>femto</td>
<td>f</td>
<td>$10^{-15}$</td>
</tr>
</tbody>
</table>
Alcohol

Portions of this monograph that are national USP text, and are not part of the harmonized text, are marked with symbols (*) to specify this fact.

C₇H₁₈O₂₃
Ethanol; Ethyl alcohol [64-17-5].

DEFINITION
Ethyl alcohol

IDENTIFICATION

Change to read:

B. SPECTROSCOPIC IDENTIFICATION TESTS (197), Infrared Spectroscopy: 197F or 197S [CN 1-May-2020]: Neat

IMPURITIES

LIMIT OF NONVOLATILE RESIDUE

Sample: 100 mL of Alcohol

Acceptance criteria: The weight of the residue is NMT 2.5 mg.

ORGANIC IMPURITIES

Sample solution A: Alcohol (substance under test)

Sample solution B: 300 µL/L of 4-methylpentan-2-ol in Sample solution A

Standard solution A: 200 µL/L of methanol in Sample solution A

Standard solution B: 10 µL/L of methanol and 10 µL/L of acetaldehyde in Sample solution A

Standard solution C: 30 µL/L of acetal in Sample solution A

Standard solution D: 2 µL/L of benzene in Sample solution A

Chromatographic system
(See Chromatography (621), System Suitability.)

Detector: Flame ionization

Column: 0.32-mm x 30-m fused-silica capillary; bonded with a 1.8-µm layer of phase G43

Split ratio: 20:1

Temperatures

Injection port: 200°

Detector: 280°

Column: See Table 1.

Table 1

<table>
<thead>
<tr>
<th>Initial Temperature (°)</th>
<th>Temperature Ramp (°/min)</th>
<th>Final Temperature (°)</th>
<th>Hold Time at Final Temperature (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>0</td>
<td>40</td>
<td>12</td>
</tr>
</tbody>
</table>

Analysis


Methanol calculation

\[
\text{Result} = \left( \frac{r_t}{r_E} \right) \times C_A
\]

Acetaldehyde calculation (sum of acetaldehyde and acetal)

\[
\text{Result} = \left[ \frac{A_E}{(A_T - A_E)} \times C_A \right] + \left[ \frac{D_T}{(D_T - D_E)} \times C_D \times (M_{r1} / M_{r2}) \right]
\]

Benzene calculation

\[
\text{Result} = \left[ \frac{B_t}{(B_T - B_E)} \times C_B \right]
\]

Any other impurity calculation

\[
\text{Result} = \left( \frac{r_t}{r_M} \right) \times C_M
\]

Table 1 (continued)

Linear velocity: 35 cm/s
Carrier gas: Helium
Injection volume: 1.0 µL
r_u = peak area of each impurity in Sample solution B
r_M = peak area of 4-methylpentan-2-ol in Sample solution B
C_M = concentration of 4-methylpentan-2-ol in Sample solution B (µL/L)

Acceptance criteria: See Table 2.

### Table 2

<table>
<thead>
<tr>
<th>Name</th>
<th>Acceptance Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>NMT 0.5, corresponding to 200 µL/L</td>
</tr>
<tr>
<td>Acetaldehyde and acetal</td>
<td>NMT 10 µL/L, expressed as acetaldehyde</td>
</tr>
<tr>
<td>Benzene</td>
<td>NMT 2 µL/L</td>
</tr>
<tr>
<td>Sum of all other impurities*</td>
<td>NMT 300 µL/L</td>
</tr>
</tbody>
</table>

*Disregard any peaks of less than 9 µL/L (0.03 times the area of the peak corresponding to 4-methylpentan-2-ol in the chromatogram obtained with Sample solution B).

### SPECIFIC TESTS

- **SPECIFIC GRAVITY (841):** 0.812–0.816 at 15.56°C, indicating 92.3%–93.8%, by weight, or 94.9%–96.0%, by volume, of C_5H_10O_2.
- **ULTRAVIOLET ABSORPTION**
  - Analytical wavelength: 235–340 nm
  - Cell: 5 cm
  - Reference: Water
  - Acceptance criteria:
    - Absorbance: NMT 0.40 at 240 nm; NMT 0.30 between 250 nm and 260 nm; NMT 0.10 between 270 nm and 340 nm
    - Curve: The spectrum shows a steadily descending curve with no observable peaks or shoulders.

### Change to read:

- **CLARITY OF SOLUTION**
  - [Note—The Sample solution is to be compared to Standard suspension A and to water in diffused daylight 5 min after preparation of Standard suspension A.] Hydrazine solution: 10 mg/mL of hydrazine sulfate in water. Allow to stand for 4–6 h.
  - Methenamine solution: Transfer 2.5 g of methenamine to a 100-mL glass-stoppered flask, add 25.0 mL of water, insert the glass stopper, and mix to dissolve.
  - Primary opalescent suspension: Transfer 25.0 mL of Hydrazine solution to the Methenamine solution in the 100-mL glass-stoppered flask. Mix, and allow to stand for 24 h. This suspension is stable for 2 months, provided it is stored in a glass container free from surface defects. The suspension must not adhere to the glass and must be well mixed before use.
  - Opalescence standard: Transfer 15.0 mL of the Primary opalescent suspension to a 1000-mL volumetric flask, and dilute with water to volume. This suspension should not be used beyond 24 h after preparation.
  - Standard suspension A: Opalescence standard and water (1 in 20)
  - Standard suspension B: Opalescence standard and water (1 in 10)

### Analysis:

- Transfer a sufficient portion of Sample solution A and Sample solution B to separate test tubes of colorless, transparent, neutral glass with a flat base and an internal diameter of 15–25 mm to obtain a depth of 40 mm.
- Similarly transfer portions of Standard suspension A, Standard suspension B, and Blank to separate matching test tubes. Compare Sample solution A, Sample solution B, Standard suspension A, Standard suspension B, and Blank in diffused daylight, viewing vertically against a black background (see Visual Comparison (630))(CN 1-May-2019). The diffusion of light must be such that Standard suspension A can readily be distinguished from water, and Standard suspension B can readily be distinguished from Standard suspension A.

### Acceptance criteria:

- **Sample solution A** and **Sample solution B** show the same clarity as that of water or their opalescence is not more pronounced than that of Standard suspension A.
- **ACIDITY OR ALKALINITY**
  - Phenolphthalein solution: Dissolve 0.1 g of phenolphthalein in 80 mL of alcohol, and dilute with water to 100 mL.
  - Sample: 20 mL of Alcohol
  - Analysis: To the Sample add 20 mL of freshly boiled and cooled water and 0.1 mL of Phenolphthalein solution. The solution is colorless. Add 1.0 mL of 0.01 N sodium hydroxide.
  - Acceptance criteria: The solution is pink (30 µL/L, expressed as acetic acid).

### ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, protected from light.
- **USP REFERENCE STANDARDS** (11)
  - USP Alcohol RS
Amantadine Hydrochloride

C<sub>9</sub>H<sub>17</sub>N · HCl 187.71
Tricyclo[3.3.1.1<sup>7</sup>]decan-1-amine, hydrochloride; 1-Adamantanamine hydrochloride [665-66-7].

**DEFINITION**
Amantadine Hydrochloride contains NLT 98.0% and NMT 102.0% of amantadine hydrochloride (C<sub>9</sub>H<sub>17</sub>N · HCl).

**IDENTIFICATION**

- **A.** *SPECTROSCOPIC IDENTIFICATION TESTS* (197), Infrared Spectroscopy: 197A, 197K, and 197S<sup>a</sup> (CN 1-May-2020)
  - Cell: 1 mm
  - Sample solution: 50 mg in 10 mL of 0.1 N hydrochloric acid, and filter. Transfer the filtrate to a suitable separator, add 1 mL of 5 N sodium hydroxide, and extract with 5 mL of methylene chloride.
  - Acceptance criteria: Meets the requirements
  - B. The retention time of the amantadine peak of the Sample solution corresponds to that of the Standard solution, as obtained in the Assay.
- **C.** IDENTIFICATION TESTS—GENERAL (191), *Chemical Identification Tests, Chloride*

**ASSAY**
- **PROCEDURE**
  - Internal standard solution: 0.3 mg/mL of adamantane in n-heptane
  - Standard stock solution: 1 mg/mL of USP Amantadine Hydrochloride RS in water
  - Standard solution: Transfer 10 mL of Standard stock solution and 10 mL of 5 N sodium hydroxide solution to a separatory funnel. Add 25 mL of Internal standard solution and shake for 10 min. Collect the upper layer of n-heptane and swirl with anhydrous sodium sulfate to remove traces of water.
  - Sample stock solution: 1 mg/mL of Amantadine Hydrochloride in water
  - Sample solution: Transfer 10 mL of Sample stock solution and 10 mL of 5 N sodium hydroxide solution to a separatory funnel. Add 25 mL of Internal standard solution and shake for 10 min. Collect the upper layer of n-heptane and swirl with anhydrous sodium sulfate to remove traces of water.

**Chromatographic system**
(See Chromatography (621), System Suitability.)
- Mode: GC
- Detector: Flame ionization
- Column: 0.53-mm × 30-m base deactivated fused-silica; coated with 1.0-µm film of stationary phase G27
- Temperatures:
  - Injection port: 220°
  - Detector: 300°
  - Column: See Table 1.

**Table 1**

<table>
<thead>
<tr>
<th>Initial Temperature (°)</th>
<th>Temperature Ramp (°/min)</th>
<th>Final Temperature (°)</th>
<th>Hold Time at Final Temperature (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>120</td>
<td>0</td>
<td>120</td>
<td>3</td>
</tr>
</tbody>
</table>

**Table 1 (continued)**

Carry gas: Helium
Flow rate: 4 mL/min
Injection volume: 2 µL
Injection type: Split ratio, 5:1 (deactivated split liner with glass wool)

**System suitability**
- **Sample:** *Standard solution*
- **Note:** The relative retention times for adamantane and amantadine are about 0.7 and 1.0, respectively.
- **Suitability requirements**
  - Relative standard deviation: NMT 1.0% for the peak response ratio of amantadine to adamantane

**Analysis**
- **Samples:** *Standard solution and Sample solution*
  - Calculate the percentage of amantadine hydrochloride (C<sub>9</sub>H<sub>17</sub>N · HCl) in the portion of Amantadine Hydrochloride taken:
  \[
  \text{Result} = \left( \frac{R_d}{R_u} \right) \times \left( \frac{C_u}{C_d} \right) \times 100
  \]
  \[
  R_d = \text{peak response ratio of amantadine to adamantane from the Sample solution}
  \]
  \[
  R_u = \text{peak response ratio of amantadine to adamantane from the Standard solution}
  \]
  \[
  C_d = \text{concentration of USP Amantadine Hydrochloride RS in the Standard stock solution (mg/mL)}
  \]
  \[
  C_u = \text{concentration of Amantadine Hydrochloride in the Sample stock solution (mg/mL)}
  \]
  - **Acceptance criteria:** 98.0%–102.0%

**IMPURITIES**
- **ORGANIC IMPURITIES**
  - **Internal standard solution:** 0.1 mg/mL of adamantane in n-heptane
  - **Peak identification solution:** 0.03 mg/mL each of USP Amantadine Related Compound A RS and USP Amantadine Related Compound B RS in Internal standard solution prepared as follows.
  - **Standard stock solution:** Transfer 25 mL of Standard stock solution and 10 mL of 5 N sodium hydroxide solution to a separatory funnel. Add 25 mL of Internal standard solution and shake for 10 min. Collect the upper layer of n-heptane and swirl with anhydrous sodium sulfate to remove traces of water.
  - **Sample stock solution:** 10 mg/mL of Amantadine Hydrochloride RS in water
  - **Standard solution:** Transfer 25 mL of Standard stock solution and 10 mL of 5 N sodium hydroxide solution to a separatory funnel. Add 25 mL of Internal standard solution and shake for 10 min. Collect the upper layer of n-heptane and swirl with anhydrous sodium sulfate to remove traces of water.

- **Chromatographic system:** Proceed as directed in the Assay.
System suitability

Sample: Standard solution

[NOTE—The relative retention times for adamantane and amantadine are 0.7 and 1.0, respectively.]

Suitability requirements

Relative standard deviation: NMT 3.0% for the peak response ratio of amantadine to adamantane

Analysis

Samples: Standard solution and Sample solution

Calculate the percentage of any individual impurity in the portion of Amantadine Hydrochloride taken:

\[ Result = \left( \frac{R_i}{R_1} \right) \times \left( \frac{C_i}{C_1} \right) \times 100 \]

where:
- \( R_i \) = peak response ratio of any individual impurity to adamantane from the Sample solution
- \( R_1 \) = peak response ratio of amantadine to adamantane from the Standard solution
- \( C_i \) = concentration of USP Amantadine Hydrochloride RS in the Standard stock solution (mg/mL)
- \( C_1 \) = concentration of Amantadine Hydrochloride in the Sample stock solution (mg/mL)

Acceptance criteria: See Table 2.

<table>
<thead>
<tr>
<th>Name</th>
<th>Relative Retention Time</th>
<th>Acceptance Criteria, NMT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amantadine</td>
<td>1.0</td>
<td>—</td>
</tr>
<tr>
<td>Amantadine Related Compound A</td>
<td>1.2</td>
<td>0.3</td>
</tr>
<tr>
<td>Amantadine Related Compound B</td>
<td>1.9</td>
<td>0.3</td>
</tr>
<tr>
<td>Any individual unspecified impurity</td>
<td>—</td>
<td>0.10</td>
</tr>
<tr>
<td>Total impurities</td>
<td>—</td>
<td>1.0</td>
</tr>
</tbody>
</table>

SPECIFIC TESTS

- **PH (791)**
  - Sample: 0.2 g/mL in water
  - Acceptance criteria: 3.0–5.5

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers.
- **USP REFERENCE STANDARDS (11)**
  - USP Amantadine Hydrochloride RS
  - USP Amantadine Related Compound A RS
    - 1-Chloroadamantane.
    - \( C_{10}H_{13}Cl \) 170.68
  - USP Amantadine Related Compound B RS
    - \( N-(Adamantan-1-yl)acetamide. \)
    - \( C_{12}H_{19}NO \) 193.29

**Amantadine Hydrochloride Capsules**

**DEFINITION**

Amantadine Hydrochloride Capsules contain NLT 95.0% and NMT 105.0% of the labeled amount of amantadine hydrochloride (\( C_{10}H_{13}N \cdot HCl \)).

**IDENTIFICATION**

**Change to read:**

- **A. SPECTROSCOPIC IDENTIFICATION TESTS (197), Infrared Spectroscopy:** 197A, 197K, or 197S (CN 1-May-2020)
  - **Cell:** 1 mm
  - **Sample solution:** Place the contents of Capsules, equivalent to 200 mg of amantadine hydrochloride, in a vessel, dissolve in 0.1 N hydrochloric acid, and filter. Transfer the filtrate to a separator, add 1 mL of 5 N sodium hydroxide, and extract with 5 mL of methylene chloride. Filter the extract through anhydrous sodium sulfate, and rinse the anhydrous sodium sulfate with 2 mL of methylene chloride.
  - **B.** The retention time of the amantadine peak of the Sample solution corresponds to that of the Standard solution, as obtained in the Assay.

**ASSAY**

**Internal standard solution:** 0.4 mg/mL of naphthalene in hexane

**Standard stock solution:** 2 mg/mL of USP Amantadine Hydrochloride RS in water

**Standard solution:** Transfer 25.0 mL of Standard stock solution into a 250-mL separator, and add 25 mL of 2 N sodium hydroxide and 50.0 mL of Internal standard solution. Shake for about 10 min, and collect the hexane layer.

**Sample stock solution:** Transfer NLT 20 Capsules to a 200-mL volumetric flask. Add 40 mL of 0.1 N hydrochloric acid and 40 mL of water. Sonicate for 20 min with intermittent shaking, and dilute with water to volume. Centrifuge the solution for 10 min and pass through a suitable filter.

**Sample solution:** Transfer 5.0 mL of the filtrate into a 250-mL separator, and add 40.0 mL of 1 N sodium hydroxide and 50.0 mL of Internal standard solution. Shake for about 10 min, and collect the hexane layer.

**Chromatographic system**

(See Chromatography (621), System Suitability.)

**Mode:** GC

**Detector:** Flame ionization

**Column:** 0.32-mm × 30-m; coated with a 0.25-µm film of phase G1

**Temperatures**

<table>
<thead>
<tr>
<th>Injection port: 250°</th>
<th>Detector: 300°</th>
<th>Column: See Table 1.</th>
</tr>
</thead>
</table>

**Table 1**

<table>
<thead>
<tr>
<th>Initial Temperature (°)</th>
<th>Temperature Ramp (°/min)</th>
<th>Final Temperature (°)</th>
<th>Hold Time at Final Temperature (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>0</td>
<td>100</td>
<td>3</td>
</tr>
<tr>
<td>100</td>
<td>10</td>
<td>200</td>
<td>2</td>
</tr>
</tbody>
</table>

**Carrier gas:** Helium

**Flow rate:** 1.4 mL/min

**Injection volume:** 2 µL

**Injection type:** Split

**Split flow rate:** 20 mL/min

**System suitability**

**Sample:** Standard solution

[NOTE—The relative retention times for naphthalene and amantadine are 0.90 and 1.0, respectively.]
Suitability requirements

Resolution: NLT 2.0 between naphthalene and amantadine
Tailing factor: NMT 2.0 for the amantadine peak
Relative standard deviation: NMT 2.0% for the peak response ratio of amantadine to naphthalene

Analysis

Samples: Standard solution and Sample solution
Calculate the percentage of the labeled amount of amantadine hydrochloride (C\textsubscript{16}H\textsubscript{23}N\textsubscript{2}·HCl) in the portion of Capsules taken:

\[
Result = \left( \frac{R_\text{a}}{R_\text{p}} \right) \times \left( \frac{C}{C_\text{u}} \right) \times 100
\]

- \( R_\text{a} \) = peak response ratio of amantadine to naphthalene from the Sample solution
- \( R_\text{p} \) = peak response ratio of amantadine to naphthalene from the Standard solution
- \( C_\text{s} \) = concentration of USP Amantadine Hydrochloride RS in the Standard solution (mg/mL)
- \( C_\text{u} \) = nominal concentration of amantadine hydrochloride in the Sample solution (mg/mL)

Acceptance criteria: 95.0%–105.0%

PERFORMANCE TESTS

- **Dissolution** (711)

  **Test 1**
  - Medium: Water; 900 mL
  - Apparatus: 1; 100 rpm
  - Time: 45 min
  - Internal standard solution: 0.054 mg/mL of naphthalene in hexane
  - Standard stock solution: 0.1 mg/mL of USP Amantadine Hydrochloride RS in water
  - Standard solution: Transfer 15.0 mL of Standard stock solution into a 50-mL screw-capped test tube, add 5.0 mL of 5 N sodium hydroxide and 10.0 mL of Internal standard solution, and shake for 60 min. Collect the hexane layer.
  - Sample solution: Transfer 15.0 mL of the filtered solution under test, and place into a 50-mL screw-capped test tube. Pipet 5.0 mL of 5 N sodium hydroxide and 10.0 mL of the Internal standard solution into the test tube, and shake for 60 min. Collect the hexane layer.

  **Chromatographic system**
  (See Chromatography (621), System Suitability.)
  - Mode: GC
  - Detector: Flame ionization
  - Column: 0.32-mm x 30-m; coated with a 0.25-µm film of phase G1
  - Temperatures
    - Injection port: 250°
    - Detector: 300°
    - Column: See Table 2.

  **Table 2**

<table>
<thead>
<tr>
<th>Initial Temperature (°)</th>
<th>Temperature Ramp (°/min)</th>
<th>Final Temperature (°)</th>
<th>Hold Time at Final Temperature (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>0</td>
<td>100</td>
<td>3</td>
</tr>
<tr>
<td>100</td>
<td>10</td>
<td>200</td>
<td>2</td>
</tr>
</tbody>
</table>

- Carrier gas: Helium
- Flow rate: 1.4 mL/min
- Injection volume: 2 µL
- Injection type: Split
- Split flow rate: 20 mL/min

System suitability

**Sample:** Standard solution

[NOTE—The relative retention times for naphthalene and amantadine are 0.90 and 1.0, respectively.]

**Suitability requirements**

- Resolution: NLT 2.0 between naphthalene and amantadine
- Tailing factor: NMT 2.0 for the amantadine peak
- Relative standard deviation: NMT 2.0% for the peak response ratio of amantadine to naphthalene

Analysis

Samples: Standard solution and Sample solution
Calculate the percentage of the labeled amount of amantadine hydrochloride dissolved:

\[
Result = \left( \frac{R_\text{a}}{R_\text{p}} \right) \times \left( \frac{C}{C_\text{u}} \right) \times V \times 100
\]

- \( R_\text{a} \) = peak response ratio of amantadine to naphthalene from the Sample solution
- \( R_\text{p} \) = peak response ratio of amantadine to naphthalene from the Standard solution
- \( C_\text{s} \) = concentration of USP Amantadine Hydrochloride RS in the Standard stock solution (mg/mL)
- \( L \) = label claim (mg/Capsule)
- \( V \) = volume of Medium, 900 mL

**Tolerances:** NLT 75% (Q) of the labeled amount of amantadine hydrochloride (C\textsubscript{16}H\textsubscript{23}N\textsubscript{2}·HCl) is dissolved.

**Test 2:** If the product complies with this test, the labeling indicates that it meets USP Dissolution Test 2.

**Medium:** Water; 900 mL

**Apparatus 2:** 75 rpm, with sinkers. [NOTE—A suitable sinker is available as catalog number CAPWHT-2S from www.qla-llc.com or www.tabletdissolution.com or www.labhut.com.]

- Time: 45 min

  **Internal standard solution:** 0.06 mg/mL of naphthalene in hexane

  **Standard stock solution:** 0.12 mg/mL of USP Amantadine Hydrochloride RS in Medium

  **Standard solution:** Transfer 60.0 mL of the Standard stock solution to a 200-mL volumetric flask. Add 20 mL of 5 N sodium hydroxide and 40.0 mL of Internal standard solution. Shake the flask for approximately 10 min, and allow the layers to separate. Use the top layer for injection.

  **Sample solution:** Transfer 3.0 mL of the solution under test to a centrifuge tube. Add 1.0 mL of 5 N sodium hydroxide and 2.0 mL of Internal standard solution. Shake the tube for approximately 10 min, and allow the layers to separate. Use the top layer for injection.

  **Chromatographic system**
  (See Chromatography (621), System Suitability.)
  - Mode: GC
  - Detector: Flame ionization
  - Column: 0.32-mm x 30-m; coated with a 0.25-µm film of phase G1
  - Temperatures
    - Injection port: 250°
    - Detector: 300°
    - Column: See Table 3.

  **Table 3**

<table>
<thead>
<tr>
<th>Initial Temperature (°)</th>
<th>Temperature Ramp (°/min)</th>
<th>Final Temperature (°)</th>
<th>Hold Time at Final Temperature (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>0</td>
<td>100</td>
<td>3</td>
</tr>
</tbody>
</table>

Published on March 26, 2020
Carrier gas: Helium
Flow rate: 4.1 mL/min
Injection volume: 2 µL
Injection type: Split
Split flow rate: 20 mL/min

Suitability requirements:
Sample: Standard solution

Resolution: NLT 2 between naphthalene and amantadine
Tailing factor: NMT 2.0 for the amantadine peak
Relative standard deviation: NMT 2.0% for the peak response ratio of amantadine to naphthalene

Analysis:
Samples: Standard solution and Sample solution

Calculate the percentage of the labeled amount of amantadine hydrochloride dissolved:
\[
\text{Result} = \left( \frac{R_U}{R_S} \times \frac{C_S}{L} \right) \times V \times 100
\]

\(R_U\) = peak response ratio of amantadine to naphthalene from the Sample solution
\(R_S\) = peak response ratio of amantadine to naphthalene from the Standard solution
\(C_S\) = concentration of USP Amantadine Hydrochloride RS in the Standard stock solution (mg/mL)
\(L\) = label claim (mg/Capsule)
\(V\) = volume of Medium, 900 mL

Tolerances: NLT 75% (Q) of the labeled amount of amantadine hydrochloride (C\(_{13}H\(_{22}\)N\cdot HCl\)) is dissolved.

• **Uniformity of Dosage Units** (905): Meet the requirements

**Impurities**

• **Organic Impurities**

Internal standard solution: 0.1 mg/mL of adamantane in n-heptane

Peak identification solution: 0.03 mg/mL each of USP Amantadine Related Compound A RS and USP Amantadine Related Compound B RS in Internal standard solution

Peak response ratio of any individual unspecified impurity to adamantane from the Sample solution:
\[
R_U = \frac{R_S}{R_S} \times \frac{C_S}{C_u} \times V \times 100
\]

\(R_U\) = peak response ratio of any individual unspecified impurity to adamantane from the Sample solution
\(R_S\) = peak response ratio of amantadine to naphthalene from the Standard solution
\(C_S\) = concentration of USP Amantadine Hydrochloride RS in the Standard stock solution (mg/mL)
\(C_u\) = nominal concentration of amantadine hydrochloride in the Sample stock solution (mg/mL)

Acceptance criteria: See Table 5.
Table 5

<table>
<thead>
<tr>
<th>Name</th>
<th>Relative Retention Time</th>
<th>Acceptance Criteria, NMT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amantadine</td>
<td>1.0</td>
<td>—</td>
</tr>
<tr>
<td>Amantadine related compound A*</td>
<td>1.2</td>
<td>—</td>
</tr>
<tr>
<td>Amantadine related compound B†</td>
<td>1.9</td>
<td>—</td>
</tr>
<tr>
<td>Any individual unspecified impurity</td>
<td>—</td>
<td>0.2</td>
</tr>
<tr>
<td>Total impurities</td>
<td>—</td>
<td>1.5</td>
</tr>
</tbody>
</table>

* Process impurity included in the table for identification only. Process impurities are controlled in the drug substance, and are not to be reported or included in the total impurities for the drug product.

**AMANTADINE HYDROCHLORIDE ORAL SOLUTION**

**DEFINITION**

Amantadine Hydrochloride Oral Solution contains NLT 95.0% and NMT 105.0% of the labeled amount of amantadine hydrochloride (C_{10}H_{17}N·HCl).

**IDENTIFICATION**

Change to read:

- **A. SPECROSCOPIC IDENTIFICATION TESTS (197), Infrared Spectroscopy**: 197A, 197K, or 197S (CN 1-May-2020)  
  Cell: 1 mm
  Sample solution: Place a volume of Oral Solution, equivalent to 200 mg of amantadine hydrochloride, in a vessel, dissolve in 0.1 N hydrochloric acid, and filter. Transfer the filtrate to a separator, add 10 mL of 0.5 N sodium hydroxide, and extract with 5 mL of methylene chloride. Filter the extract through anhydrous sodium sulfate, and rinse the anhydrous sodium sulfate with 2 mL of methylene chloride.
  Acceptance criteria: Meets the requirements
  - B. The retention time of the major peak of the Sample solution corresponds to that of the Standard solution, as obtained in the Assay.

**ASSAY**

- **PROCEDURE**
  - Internal standard solution: 0.3 mg/mL of adamantane in n-heptane
  - Standard stock solution: 1 mg/mL of USP Amantadine Hydrochloride RS in water

**Standard solution**: Transfer 10 mL of Standard stock solution to a separatory funnel and add 10 mL of 5 N sodium hydroxide solution. Add 25 mL of Internal standard solution and shake for 10 min. Collect the n-heptane upper layer and swirl with anhydrous sodium sulfate to remove traces of water.

**Sample stock solution**: Nominally 1 mg/mL of amantadine hydrochloride from a portion of Oral Solution in water

**Sample solution**: Transfer 10 mL of Sample stock solution to a separatory funnel and add 10 mL of 5 N sodium hydroxide solution. Add 25 mL of Internal standard solution and shake for 10 min. Collect the n-heptane upper layer and swirl with anhydrous sodium sulfate to remove traces of water.

**Chromatographic system**

(See Chromatography (621), System Suitability.)

**Mode**: GC

**Detector**: Flame ionization

**Column**: 0.53-mm × 30-m base deactivated fused-silica; coated with 1.0-µm film of stationary phase G27

**Temperatures**

- **Injection port**: 220°C

**Detector**: 300°C

**Column**: See Table 1.

<table>
<thead>
<tr>
<th>Table 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Temperature (°C)</td>
</tr>
<tr>
<td>---------</td>
</tr>
<tr>
<td>120</td>
</tr>
<tr>
<td>120</td>
</tr>
</tbody>
</table>

**Carrier gas**: Helium

**Flow rate**: 4 mL/min

**Injection volume**: 2 µL

**Injection type**: Split ratio, 5:1 (deactivated split liner with glass wool)

**System suitability**

Sample: Standard solution

[NOTE—The relative retention times for adamantane and amantadine are about 0.7 and 1.0, respectively.]

**Suitability requirements**

- **Tailing factor**: NMT 2.0 for the amantadine peak
- **Relative standard deviation**: NMT 2.0% for the peak response ratio of amantadine to adamantane

**Analysis**

Samples: Standard solution and Sample solution

Calculate the percentage of the labeled amount of amantadine hydrochloride (C_{10}H_{17}N·HCl) in the portion of Oral Solution taken:

Result = \((R_u/R_a) \times (C_u/C_a)\) \times 100

- **R_u** = peak response ratio of amantadine to adamantane from the Sample solution
- **R_a** = peak response ratio of amantadine to adamantane from the Standard solution
- **C_u** = concentration of USP Amantadine Hydrochloride RS in the Standard stock solution (mg/mL)
- **C_a** = nominal concentration of amantadine hydrochloride in the Sample stock solution (mg/mL)

**Acceptance criteria**: 95.0%–105.0%
**IMPURITIES**

- **ORGANIC IMPURITIES**

  **Internal standard solution:** 0.1 mg/mL of adamantane in n-heptane

  **Peak identification solution:** 0.03 mg/mL each of USP Amantadine Related Compound A RS and USP Amantadine Related Compound B RS in Internal standard solution prepared as follows. Transfer suitable amounts of USP Amantadine Related Compound A RS and USP Amantadine Related Compound B RS to a suitable volumetric flask. Add methylene chloride to about 5% of the flask volume to dissolve, and dilute with Internal standard solution to volume.

  **Standard stock solution:** 0.03 mg/mL of USP Amantadine Hydrochloride RS in water

  **Standard solution:** Transfer 25 mL of Standard stock solution, 10 mL of 5 N sodium hydroxide solution, and 25 mL of Internal standard solution to a separatory funnel and shake for 10 min. Collect the upper layer of n-heptane and swirl with anhydrous sodium sulfate to remove traces of water.

  **Sample stock solution:** Nominally 10.0 mg/mL of amantadine hydrochloride from Oral Solution in water

  **Sample solution:** Transfer 25 mL of Sample stock solution, equivalent to 250 mg of amantadine hydrochloride, and 10 mL of 5 N sodium hydroxide solution to a separatory funnel. Add 25 mL of Internal standard solution and shake for 10 min. Collect the upper layer of n-heptane and swirl with anhydrous sodium sulfate to remove traces of water.

  **Chromatographic system:** Proceed as directed in the Assay.

  **System suitability**

  **Sample:** Standard solution

  **Suitability requirements**

  **Relative standard deviation:** NMT 3.0% for the peak response ratio of amantadine and adamantane

  **Analysis**

  **Samples:** Standard solution and Sample solution

  Calculate the percentage of any individual unspecified impurity in the portion of Oral Solution taken:

  \[
  \text{Result} = \frac{R_u / R_s}{C_i / C_u} \times 100
  \]

  where:

  \[R_u\] = peak response ratio of any individual unspecified impurity to adamantane from the Sample solution

  \[R_s\] = peak response ratio of amantadine to adamantane from the Standard solution

  \[C_i\] = concentration of USP Amantadine Hydrochloride RS in the Standard stock solution (mg/mL)

  \[C_u\] = nominal concentration of amantadine hydrochloride in the Sample stock solution (mg/mL)

  **Acceptance criteria:** see Table 2.

<table>
<thead>
<tr>
<th>Name</th>
<th>Relative Retention Time</th>
<th>Acceptance Criteria NMT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amantadine</td>
<td>1.0</td>
<td>—</td>
</tr>
<tr>
<td>Amantadine related compound A*</td>
<td>1.2</td>
<td>—</td>
</tr>
<tr>
<td>Amantadine related compound B*</td>
<td>1.9</td>
<td>—</td>
</tr>
</tbody>
</table>

*Process impurity included in the table for identification only. Process impurities are controlled in the drug substance, and are not to be reported or included in the total impurities for the drug product.

**ADDITIONAL REQUIREMENTS**

- **PACKAGING AND STORAGE:** Preserve in tight containers. Store at controlled room temperature.

- **USP REFERENCE STANDARDS** (11)

  USP Amantadine Hydrochloride RS
  USP Amantadine Related Compound A RS
  1-Chloroadamantane.
  C10H14Cl 170.68
  USP Amantadine Related Compound B RS
  N-(Adamantan-1-yl)acetamide.
  C16H24NO 193.29

---

**Amiodarone Hydrochloride**

**Change to read:**

\[\text{C}_{25}\text{H}_{32}\text{NO}_{3} \cdot \text{HCl}\]

681.77
Methanone, (2-buty1-3-benzofuranyl)4-[2-(diethylamino)ethoxy]-3,5-diiodophenyl-1-hydrochloride; 2-Butyl-3-benzofuranyl 4-[2-(diethylamino)ethoxy]-3,5-diiodophenyl ketone hydrochloride [19774-82-4].
2-Butyl-3-benzofuranyl 4-[2-(diethylamino)ethoxy]-3,5-diiodophenyl ketone [1957-25-3].

**DEFINITION**

Amiodarone Hydrochloride contains NLT 98.5% and NMT 101.0% of C25H32NO3·HCl, calculated on the dried basis.

**IDENTIFICATION**

- **A. INFRARED ABSORPTION** (197K)

- **B. IDENTIFICATION TESTS—GENERAL, Chloride** (191): Meets the requirements

**ASSAY**

**PROCEDURE**

Buffer: Dissolve 6.80 g of monobasic potassium phosphate in 900 mL of water, and add 1.0 mL of triethylamine. Adjust with phosphoric acid to a pH of 6.00 ± 0.05, and dilute with water to 1000 mL.

Diluent: Acetonitrile and water (1:1)

Mobile phase: Acetonitrile and Buffer (1:1)

**Standard stock solution:** 0.5 mg/mL of USP Amiodarone Hydrochloride RS in methanol

**Standard solution:** 0.1 mg/mL USP Amiodarone Hydrochloride RS in Diluent from Standard stock solution

**Sample stock solution:** 0.5 mg/mL of Amiodarone Hydrochloride in methanol

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Published on March 26, 2020
Sample solution: 0.1 mg/mL of Amiodarone Hydrochloride in Diluent from Sample stock solution

Chromatographic system
(See Chromatography (621), System Suitability.)
Mode: LC
Detector: UV 240 nm
Column: 3.9-mm × 15-cm; 5-µm packing L26
Flow rate: 1.5 mL/min
Injection size: 10 µL

System suitability
Sample: Standard solution
Suitability requirements
Column efficiency: NLT 1000 theoretical plates
Tailing factor: NMT 2.0
Relative standard deviation: NMT 1.0%

Analysis
Samples: Standard solution and Sample solution
Calculate the percentage of C₂₉H₃₂I₃NO₃ · HCl in the portion of Amiodarone Hydrochloride taken:

\[
\text{Result} = \left( \frac{r_u}{r_S} \right) \times \left( \frac{C_u}{C_S} \right) \times 100
\]

- \( r_u \): peak response of amiodarone in the Sample solution
- \( r_S \): peak response of amiodarone in the Standard solution
- \( C_S \): concentration of USP Amiodarone Hydrochloride RS in the Standard solution (mg/mL)
- \( C_u \): nominal concentration of Amiodarone Hydrochloride in the Sample solution (mg/mL)

Acceptance criteria: 98.5%–101.0%, on the dried basis

IMPURITIES
INORGANIC IMPURITIES
• Residue on Ignition (281): NMT 0.1% on a 1-g sample

ORGANIC IMPURITIES
[NOTE—The product meets the requirements for both Procedure 1 and Procedure 2.]

• Procedure 1
Potassium iodosubnitrate solution: Dissolve 100 g of tartaric acid in 400 mL of water, and add 8.5 g of bismuth subnitrate. Shake for 1 h, add 200 mL of a 400 g/L solution of potassium iodide, and shake well. Allow to stand for 24 h, filter, and protect from light.

Standard solution A: 0.02 mg/mL of USP Amiodarone Related Compound H RS in methylene chloride
Standard solution B: Standard solution A and Sample solution (1:1).

Sample solution: 100 mg/mL of Amiodarone Hydrochloride in methylene chloride

Chromatographic system
(See Chromatography (621), Thin-Layer Chromatography.)
Mode: TLC
Adsorbent: Suitable layer of chromatographic silica gel and fluorescent indicator with maximum absorbance at 254 nm

Application volume
Standard solution A: 50 µL
Standard solution B: 100 µL
Sample solution: 50 µL

Developing solvent system: Methylene chloride, methanol, and anhydrous formic acid (17:2:1)

Analysis
Samples: Standard solution A, Standard solution B, and Sample solution
Develop the plate in the Developing solvent system until the solvent front has moved NLT two-thirds the length of the plate, and dry in a current of cold air. Spray the plate with Potassium iodosubnitrate solution and then with 3% hydrogen peroxide solution. Examine immediately in daylight: the spot from Standard solution B due to amiodarone related compound H is clearly visible.

Acceptance criteria: Any spot with the same \( R_f \) as the spot due to amiodarone related compound H from the Sample solution is not more intense than the spot from Standard solution A (0.02%).

• Procedure 2
Buffer: Add 3 mL of glacial acetic acid to 800 mL of water. Adjust with diluted ammonia solution to a pH of 4.9, and dilute with water to 1000 mL.
Diluent: Acetonitrile and water (1:1)

Standard stock solution: Dissolve equal quantities of USP Amiodarone Related Compound D RS, USP Amiodarone Related Compound E RS, and USP Amiodarone Hydrochloride RS in a known amount of methanol.

Standard solution: 5 mg/mL of Amiodarone Hydrochloride in Diluent

Chromatographic system
(See Chromatography (621), System Suitability.)
Mode: LC
Detector: UV 240 nm
Column: 4.6-mm × 15-cm; 5-µm packing L1
Column temperature: 30°
Flow rate: 1 mL/min
Injection size: 10 µL
Run time: 2 times the retention time of amiodarone

System suitability
Sample: Standard solution
Suitability requirements
Resolution: NLT 3.5 between amiodarone related compound D and amiodarone related compound E

Analysis
[NOTE—Disregard any peak that is less than 0.05%.

Samples: Standard solution and Sample solution
Calculate the percentage of each impurity in the portion of Amiodarone Hydrochloride taken:

\[
\text{Result} = \left( \frac{r_i}{r_S} \right) \times \left( \frac{C_i}{C_S} \right) \times 100
\]

- \( r_i \): peak response of each impurity in the Sample solution
- \( r_S \): peak response of amiodarone in the Standard solution
- \( C_i \): concentration of USP Amiodarone Hydrochloride RS in the Standard solution (mg/mL)
- \( C_u \): nominal concentration of Amiodarone Hydrochloride in the Sample solution (mg/mL)

Acceptance criteria
Individual impurities: See Impurity Table I.
Total impurities: NMT 0.5%

<table>
<thead>
<tr>
<th>Impurity Table I</th>
<th>Name</th>
<th>Relative Retention Time</th>
<th>Acceptance Criteria, NMT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amiodarone related compound A'</td>
<td>0.26</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>Amiodarone related compound D'</td>
<td>0.29</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>Amiodarone related compound E'</td>
<td>0.37</td>
<td>0.2</td>
<td></td>
</tr>
</tbody>
</table>

Published on March 26, 2020
**Impurity Table 1 (continued)**

<table>
<thead>
<tr>
<th>Name</th>
<th>Relative Retention Time</th>
<th>Acceptance Criteria, NMT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amiodarone related compound B&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.49</td>
<td>0.2</td>
</tr>
<tr>
<td>Amiodarone related compound C&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.55</td>
<td>0.2</td>
</tr>
<tr>
<td>Amiodarone related compound G&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.62</td>
<td>0.2</td>
</tr>
<tr>
<td>Amiodarone related compound F&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.69</td>
<td>0.2</td>
</tr>
<tr>
<td>Amiodarone hydrochloride</td>
<td>1.00</td>
<td>—</td>
</tr>
<tr>
<td>Any other individual impurity</td>
<td>—</td>
<td>0.10</td>
</tr>
</tbody>
</table>

<sup>a</sup> (2-Butylbenzofuran-3-yl)[4-[2-(diethylamino)ethoxy]phenyl]methanone.
<sup>b</sup> (2-Butylbenzofuran-3-yl)[4-hydroxy-3,5-diiodophenyl]methanone.
<sup>c</sup> (2-Butylbenzofuran-3-yl)[4-hydroxy-2-(ethy lamino)ethoxy]-3,5-diiodophenyl methanone.
<sup>d</sup> (2-Butylbenzofuran-3-yl)[4-[2-(ethylamino)ethoxy]-3,5-diiodophenyl]methanone.
<sup>e</sup> (2-Butylbenzofuran-3-yl)[4-[2-(diethylamino)ethoxy]-3-iodophenyl]methanone.
<sup>f</sup> [2-(1RS)-1-Methoxybutyl]benzofuran-3-yl[[4-[2-(diethylamino)ethoxy]-3,5-di iodophenyl]methanone.
<sup>g</sup> (2-Butylbenzofuran-3-yl)[4-hydroxy-3,5-diiodophenyl]methanone.

**SPECIFIC TESTS**

**LIMIT OF IODIDES**

**Solution A:** Add 1.50 g of Amiodarone Hydrochloride to 40 mL of water at 80°, and shake until completely dissolved. Cool, and dilute with water to 50.0 mL.

**Standard solution:** To 15.0 mL of Solution A add 1.0 mL of 0.1 M hydrochloric acid, 1.0 mL of an 88.2 mg/L solution of potassium iodide, and 1.0 mL of 0.05 M potassium iodate. Dilute with water to 20.0 mL. Allow to stand protected from light for 4 h.

**Sample solution:** To 15.0 mL of Solution A add 1.0 mL of 0.1 M hydrochloric acid and 1.0 mL of 0.05 M potassium iodate. Dilute with water to 20.0 mL. Allow to stand protected from light for 4 h.

**Analysis:** Measure the absorbances of the Standard solution and the Sample solution at 420 nm, using a mixture of 15.0 mL of Solution A and 1.0 mL of 0.1 M hydrochloric acid diluted with water to 20.0 mL to serve as the blank. The absorbance of the Sample solution is NMT half the absorbance of the Standard solution.

**Acceptance criteria:** NMT 150 ppm

- **pH (791):** 3.2–3.8. Dissolve 1 g of Amiodarone Hydrochloride in water by heating at 80°. Cool, and dilute with water to 20 mL.

- **Loss on Drying (731):** Use 1 g of sample, and dry under vacuum (NMT 0.3 kPa) at 50° for 4 h: it loses NMT 0.5% of its weight.

**ADDITIONAL REQUIREMENTS**

- **Packaging and Storage:** Preserve in light-resistant, tight containers. Store at controlled room temperature.

- **USP Reference Standards (11):**
  - USP Amiodarone Hydrochloride RS
  - USP Amiodarone Related Compound D RS
    - (2-Butylbenzofuran-3-yl)(4-hydroxy-3,5-diiodophenyl) methanone. 
    - \(C_{19}H_{16}I_3O_3\) 546.14
  - USP Amiodarone Related Compound E RS
    - (2-Butylbenzofuran-3-yl)(4-hydroxyphenyl) methanone. 
    - \(C_{19}H_{18}O_3\) 294.34
  - USP Amiodarone Related Compound H RS
    - 2-Chloro-N,N-diethylethanamine. 
    - \(C_2H_4ClN\) 135.64

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**Amiodarone Hydrochloride Injection**

**DEFINITION**

Amiodarone Hydrochloride Injection is a sterile solution of Amiodarone Hydrochloride. It contains NLT 90.0% and NMT 110.0% of the labeled amount of amiodarone hydrochloride \((C_{25}H_{36}I_2NO_3 \cdot HCl)\). It may contain suitable preservatives.

**IDENTIFICATION**

- **A.** The retention time of the major peak of the Sample solution corresponds to that of the Standard solution, as obtained in the Assay.

**Add the following:**

- **B.** The UV spectrum of the major peak of the Sample solution corresponds to that of the Standard solution, as obtained in the Assay.<sup>▲</sup>

**ASSAY**

**Change to read:**

- **Procedure:** Buffer: 1.36 g/L of potassium phosphate, monobasic in water prepared as follows. To 1.36 g of potassium phosphate, monobasic in a 1-L volumetric flask add about 900 mL of water and 1 mL of triethylamine. Adjust with phosphoric acid to a pH of 6.0. Dilute with water to volume.

**Mobile phase:** Acetonitrile and Buffer (80:20)

**Diluent:** Acetonitrile and water (60:40)

**Standard solution:** 0.025 mg/mL of USP Amiodarone Hydrochloride RS in Diluent

**Sample solution:** Nominally 0.025 mg/mL of amiodarone hydrochloride in Diluent from a suitable volume of Injection

**Chromatographic system**

(See Chromatography (621), System Suitability.)

- **Mode:** LC

- **Detector:** UV 240 nm. For Identification B, use a diode array detector in the range of 200–400 nm.<sup>▲</sup>

- **Column:** 4.6-mm × 10-cm; 5-µm packing L1

- **Flow rate:** 2 mL/min

- **Injection volume:** 20 µL

- **Run time:** NLT 2 times the retention time of amiodarone

**System suitability**

- **Sample:** Standard solution

**Suitability requirements**

- **Tailing factor:** NMT 2.0

- **Relative standard deviation:** NMT 2.0%

**Analysis**

- **Samples:** Standard solution and Sample solution

Calculate the percentage of the labeled amount of amiodarone hydrochloride \((C_{25}H_{36}I_2NO_3 \cdot HCl)\) in the portion of Injection taken:

\[
\text{Result} = \left( \frac{r_U}{r_S} \right) \times \left( \frac{C_S}{C_U} \right) \times 100
\]

\(r_U\) = peak response<sup>▲</sup> of amiodarone from the Sample solution

\(r_S\) = peak response<sup>▲</sup> of amiodarone from the Standard solution

\(C_S\) = concentration of USP Amiodarone Hydrochloride RS in the Standard solution (mg/mL)

\(C_U\) = nominal concentration of amiodarone hydrochloride in the Sample solution (mg/mL)
Acceptance criteria: 90.0%–110.0%

OTHER COMPONENTS

Change to read:

• CONTENT OF BENZYL ALCOHOL (if present)
  Internal standard solution: 1 mg/mL of phenol in isopropyl alcohol
  Standard stock solution: \( 1.6 \text{ of (USP 1-Dec-2019)} \) mg/mL of USP Benzyl Alcohol RS in isopropyl alcohol
  Standard solution: \( 0.2 \text{ mg/mL of phenol and 0.19 mg/mL of USP Benzyl Alcohol RS in isopropyl alcohol from the Internal standard solution and the Standard stock solution (USP 1-Dec-2019)} \)
  Sample stock solution: Nominally \( 1.6 \text{ (USP 1-Dec-2019)} \) mg/mL of benzyl alcohol in isopropyl alcohol*
  Blank: 0.2 mg/mL of phenol in isopropyl alcohol*

Chromatographic system
(See Chromatography (621), System Suitability.)

Mode: GC
Detector: Flame ionization
Column: 0.32-mm × 30-m fused silica capillary; coated with 1-µm film of fused silica (USP 1-Dec-2019) phase G16

Temperatures
  Injection port: 200°
  Detector: 200°
  Carrier gas: Nitrogen
  Flow rate: 10 mL/min
  Injection volume: 1 µL
  Injection type: Split; split ratio, 10:1

System suitability
Sample: Standard solution
Suitability requirements
Relative standard deviation: NMT 2.0% for the peak response ratio of benzyl alcohol to phenol

Analysis
Samples: Standard solution and Sample solution
Calculate the percentage of the labeled amount of benzyl alcohol in the portion of injection taken:

\[
\text{Result} = \left( \frac{R_U}{R_S} \right) \times \left( \frac{C_S}{C_U} \right) \times 100
\]

\( R_U \) = peak response ratio of benzyl alcohol to phenol from the Sample solution
\( R_S \) = peak response ratio of benzyl alcohol to phenol from the Standard solution
\( C_S \) = concentration of USP Benzyl Alcohol RS in the Standard solution (mg/mL)
\( C_U \) = nominal concentration of benzyl alcohol in the Sample solution (mg/mL)

Acceptance criteria: 90.0%–110.0%

IMPURITIES

Change to read:

• LIMIT OF IODIDE
  Use freshly prepared solutions in amber glassware.
**Sample solution:** Nominally 1 mg/mL of amiodarone hydrochloride in Diluent from a suitable volume of Injection

**Chromatographic system**
(See Chromatography (621), System Suitability.)

**Mode:** LC

**Detector:** UV 240 nm

**Column:** 4.6-mm x 15-cm; 5-µm packing L1

**Temperatures**
- Autosampler: 2°-8° (USP 1-Dec-2019)
- Column: 30°

**Flow rate:** 1 mL/min

**Injection volume:** 10 µL

**Run time:** NLT 1.5 times the retention time of amiodarone for the Standard solution, and NLT 2 times the retention time of amiodarone for the Sample solution

**System suitability**

**Sample:** Standard solution for relative retention times.

[NOTE—See Table 1 for relative retention times.]

**Suitability requirements**

- **Resolution:** NLT 3.5 between the amiodarone related compound D and amiodarone related compound E peaks (USP 1-Dec-2019)
- **Tailing factor:** NMT 2.0 for the amiodarone, amiodarone related compound D, and amiodarone related compound E peaks (USP 1-Dec-2019)
- **Relative standard deviation:** NMT 5.0% for the amiodarone, amiodarone related compound D, and amiodarone related compound E peaks (USP 1-Dec-2019)

**Analysis**

**Samples:** Standard solution and Sample solution

Calculate the percentage of amiodarone related compound D or amiodarone related compound E in the portion of Injection taken:

\[
\text{Result} = \left( \frac{r_I}{r_S} \right) \times \left( \frac{C_J}{C_U} \right) \times 100
\]

where:
- \( r_I \) = peak response (USP 1-Dec-2019) of amiodarone related compound D or amiodarone related compound E from the Sample solution
- \( r_S \) = peak response (USP 1-Dec-2019) of amiodarone related compound D or amiodarone related compound E from the Standard solution
- \( C_J \) = concentration of USP Amiodarone Related Compound D RS or USP Amiodarone Related Compound E RS in the Standard solution (mg/mL)
- \( C_U \) = nominal concentration of amiodarone hydrochloride in the Sample solution (mg/mL)

Calculate the percentage of any unspecified degradation product in the portion of Injection taken:

\[
\text{Result} = \left( \frac{r_I}{r_S} \right) \times \left( \frac{C_J}{C_U} \right) \times 100
\]

where:
- \( r_I \) = peak response (USP 1-Dec-2019) of any unspecified degradation product from the Sample solution
- \( r_S \) = peak response (USP 1-Dec-2019) of amiodarone from the Standard solution (USP 1-Dec-2019)
- \( C_J \) = concentration of USP Amiodarone Hydrochloride RS in the Standard solution (mg/mL)
- \( C_U \) = nominal concentration of amiodarone hydrochloride in the Sample solution (mg/mL)

**Acceptance criteria:** See Table 1.

**SPECIFIC TESTS**

- **Bacterial Endotoxins Test (85):** Meets the requirements (USP 1-Dec-2019)
- **Sterility Tests (71):** Meets the requirements
- **pH (791):** 3.0–5.0
- **Particulate Matter in Injections (788):** Meets the requirements for small-volume injections
- **Other Requirements:** Meets the requirements in Injections and Implanted Drug Products (1)

**ADDITIONAL REQUIREMENTS**

- **Packaging and Storage:** Preserve in single-dose or multiple-dose glass containers, protected from light and excessive heat. Store at controlled room temperature.
- **Labeling:** Label it to indicate that it is to be diluted to the appropriate strength with a suitable parenteral vehicle prior to administration. Label it to indicate the type and amount of preservative used. Label it to indicate that it is preservative free, if no preservative is present.
- **USP Reference Standards (11)**
  - USP Amiodarone Hydrochloride RS
  - USP Amiodarone Related Compound D RS
    - (2-Butylbenzofuran-3-yl)(4-hydroxy-3,5-diiodophenyl)methanone.
    - \( C_{11}H_{13}I_3O_3 \) 546.14
  - USP Amiodarone Related Compound E RS
    - (2-Butylbenzofuran-3-yl)(4-hydroxyphenyl)methanone.
    - \( C_{11}H_{12}O_3 \) 294.34
  - USP Benzyl Alcohol RS

Add the following:

**Amiodarone Hydrochloride Tablets**

**DEFINITION**

Amiodarone Hydrochloride Tablets contain NLT 90.0% and NMT 110.0% of the labeled amount of amiodarone hydrochloride (C₂₂H₂₃I₂NO₃ · HCl).

**IDENTIFICATION**

- **A.** The retention time of the major peak of the Sample solution corresponds to that of the Standard solution, as obtained in the Assay.
• B. The UV spectrum of the major peak of the Sample solution corresponds to that of the Standard solution, as obtained in the Assay.

ASSAY

• PROCEDURE

Buffer: Add 3 mL of acetic acid, glacial to 1 L of water. Adjust with ammonia water, 25 percent to a pH of 3.0.

Mobile phase: Acetonitrile and Buffer (40:60)

Standard solution: 0.1 mg/mL of USP Amiodarone Hydrochloride RS in Mobile phase

Sample stock solution: Nominally 1 mg/mL of amiodarone hydrochloride in Mobile phase prepared as follows:

Transfer a quantity, equivalent to 100 mg of amiodarone hydrochloride, from NLT 20 finely powdered Tablets to a 100-mL volumetric flask. Add Mobile phase to about 50% of the final flask volume. Sonicate with occasional shaking to dissolve. Cool the solution and dilute with Mobile phase to volume.

Sample solution: Nominally 0.1 mg/mL of amiodarone hydrochloride in Mobile phase from Sample stock solution. Pass a portion of the solution through a suitable filter of 0.45-µm pore size, discard the first few milliliters, and collect the filtrate.

Chromatographic system

(See Chromatography (621), System Suitability.)

Mode: LC

Detector: UV 240 nm. For Identification B, use a diode array detector in the range of 200–400 nm.

Column: 4.6-mm × 25-cm; 5-µm packing L1

Column temperature: 30°

Flow rate: 1 mL/min

Injection volume: 10 µL

Run time: NLT 2.5 times the retention time of amiodarone

System suitability

Sample: Standard solution

Suitability requirements

Tailing factor: NMT 2.0

Relative standard deviation: NMT 2.0%

Analysis

Samples: Standard solution and Sample solution

Calculate the percentage of the labeled amount of amiodarone hydrochloride (C23H24I2NO3 · HCl) dissolved in the portion of Tablets taken:

\[
\text{Result} = \left( \frac{r_u}{r_S} \right) \times \left( \frac{C_u}{C_S} \right) \times 100
\]

where

- \( r_u \) = peak response of amiodarone from the Sample solution
- \( r_S \) = peak response of amiodarone from the Standard solution
- \( C_u \) = concentration of USP Amiodarone Hydrochloride RS in the Standard solution (mg/mL)
- \( C_S \) = nominal concentration of amiodarone hydrochloride in the Sample solution (mg/mL)

Acceptance criteria: 90.0%–110.0%

PERFORMANCE TESTS

Change to read:

• DISSOLUTION (711)

*Test 1A (88 1-Dec-2019)

Medium: 19% (w/v) sodium dodecyl sulfate; 1000 mL

Apparatus 2: 100 rpm

Time: 60 min

Standard stock solution: 0.2 mg/mL of USP Amiodarone Hydrochloride RS prepared as follows. Transfer an appropriate quantity of USP Amiodarone Hydrochloride RS to a suitable volumetric flask and add methanol to 5% of the final flask volume. Sonicate to dissolve and dilute with Medium to volume.

Standard solution: 0.01 mg/mL of USP Amiodarone Hydrochloride RS in Medium from Standard stock solution

Sample solution: Dilute a portion of the solution under test with Medium to a concentration similar to that of the Standard solution. Pass a portion of the solution through a suitable filter of 0.45-µm pore size, discard the first few milliliters, and collect the filtrate.

Instrumental conditions

(See Ultraviolet-Visible Spectroscopy (857).)

Mode: UV

Analytical wavelength: 243 nm

Cell: 1 cm

Blank: Medium

Analysis

Samples: Standard solution and Sample solution

Calculate the percentage of the labeled amount of amiodarone hydrochloride (C23H24I2NO3 · HCl) dissolved:

\[
\text{Result} = \left( \frac{A_u}{A_S} \right) \times C_2 \times D \times V \times \left( \frac{1}{L} \right) \times 100
\]

where

- \( A_u \) = absorbance of amiodarone from the Sample solution
- \( A_S \) = absorbance of amiodarone from the Standard solution
- \( C_2 \) = concentration of USP Amiodarone Hydrochloride RS in the Standard solution (mg/mL)
- \( D \) = dilution factor for the Sample solution
- \( V \) = volume of Medium, 1000 mL
- \( L \) = label claim of amiodarone hydrochloride (mg/Tablet)

Tolerances: NLT 80% (Q) of the labeled amount of amiodarone hydrochloride (C23H24I2NO3 · HCl) is dissolved.

*Test 2: If the product complies with this test, the labeling indicates that it meets USP Dissolution Test 2.

Medium: 0.2% (v/v) polysorbate 80 in 0.05 N hydrochloric acid prepared as follows. Add 26 mL of hydrochloric acid and 12 mL of polysorbate 80 to 6 L of deaerated water; 900 mL

Apparatus 2: 75 rpm

Time: 30 min

Standard solution: 0.22 mg/mL of USP Amiodarone Hydrochloride RS prepared as follows. Transfer an appropriate quantity of USP Amiodarone Hydrochloride RS to a suitable volumetric flask, and add methanol to 20% of the final flask volume. Sonicate to dissolve and dilute with Medium to volume.

Sample solution: Pass a portion of the solution through a suitable filter of 0.45-µm pore size, discard the first few milliliters, and collect the filtrate.

Instrumental conditions

Mode: UV

Analytical wavelength: 244 nm

Cell: 0.1 cm

Blank: Medium

Analysis

Samples: Standard solution and Sample solution
Calculate the percentage of the labeled amount of amiodarone hydrochloride (C\textsubscript{25}H\textsubscript{33}I\textsubscript{2}N\textsubscript{3}O\textsubscript{7} \cdot HCl) dissolved:

\[
\text{Result} = (A_0/A_s) \times C_s \times V \times (1/L) \times 100
\]

\(A_0\) = absorbance of amiodarone from the Sample solution
\(A_s\) = absorbance of amiodarone from the Standard solution
\(C_s\) = concentration of USP Amiodarone Hydrochloride RS in the Standard solution (mg/mL)
\(V\) = volume of Medium, 900 mL
\(L\) = label claim of amiodarone hydrochloride (mg/Tablet)

Tolerances: NLT 75% (Q) of the labeled amount of amiodarone hydrochloride (C\textsubscript{25}H\textsubscript{33}I\textsubscript{2}N\textsubscript{3}O\textsubscript{7} \cdot HCl) is dissolved. ▲ (RB 1-Dec-2019)

**UNIFORMITY OF DOSAGE UNITS (905):** Meet the requirements

**IMPURITIES**

**ORGANIC IMPURITIES**

**Buffer:** Add 3 mL of acetic acid, glacial to 800 mL of water. Adjust with 10% (v/v) ammonia hydroxide solution to a pH of 4.9. Dilute with water to 1000 mL.

**Mobile phase:** Acetonitrile, methanol, and Buffer (40:30:30)

**Diluent:** Acetonitrile and water (50:50)

**Standard solution:** 0.01 mg/mL of USP Amiodarone Hydrochloride RS in Diluent

**Sensitivity solution:** 0.3 µg/mL of USP Amiodarone Hydrochloride RS in Diluent from Standard solution

**Sample solution:** Nominally 1 mg/mL of amiodarone hydrochloride in Diluent prepared as follows. Transfer a quantity equivalent to 50 mg of amiodarone hydrochloride from NLT 20 finely powdered Tablets to a 50-mL volumetric flask. Add Diluent to 50% of the final flask volume. Sonicate with occasional shaking to dissolve. Cool the solution and dilute with Diluent to volume. Pass a portion of the solution through a suitable filter of 0.45-µm pore size, discard the first few milliliters, and collect the filtrate.

**Chromatographic system**

(See Chromatography (621), System Suitability.)

Mode: LC

Detector: UV 240 nm

Column: 4.6-mm × 15-cm; 5-µm packing L1

Column temperature: 30°

Flow rate: 1 mL/min

Injection volume: 10 µL

Run time: NLT 1.7 times the retention time of amiodarone for the Standard solution; NLT 3.4 times the retention time of amiodarone for the Sample solution

**System suitability**

Samples: Standard solution and Sensitivity solution

Suitability requirements

Relative standard deviation: NMT 10.0%, Standard solution

Signal-to-noise ratio: NLT 10, Sensitivity solution

Analysis

Samples: Standard solution and Sample solution

Calculate the percentage of amiodarone related compound D or any unspecified degradation product in the portion of Tablets taken:

\[
\text{Result} = (r_0/r_s) \times (C_s/C_0) \times (1/F) \times 100
\]

**Acceptance criteria:** See Table 1. The reporting threshold is 0.03%.

<table>
<thead>
<tr>
<th>Name</th>
<th>Relative Retention Time</th>
<th>Relative Response Factor</th>
<th>Acceptance Criteria, NMT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amiodarone related compound A(^a)(^b)</td>
<td>0.22</td>
<td>=</td>
<td>=</td>
</tr>
<tr>
<td>Amiodarone related compound D(^c)</td>
<td>0.29</td>
<td>0.90</td>
<td>0.5</td>
</tr>
<tr>
<td>Amiodarone related compound C(^d)(^b)</td>
<td>0.52</td>
<td>=</td>
<td>=</td>
</tr>
<tr>
<td>Amiodarone</td>
<td>1.00</td>
<td>=</td>
<td>=</td>
</tr>
<tr>
<td>Any unspecified degradation product</td>
<td></td>
<td>1.00</td>
<td>0.2</td>
</tr>
<tr>
<td>Total degradation products</td>
<td></td>
<td>=</td>
<td>1.0</td>
</tr>
</tbody>
</table>

\(^a\)(2-Butylbenzofuran-3-yl){4-[2-(diethylamino)ethoxy]phenyl}methanone.

\(^b\)Process impurity included in the table for identification only. Process impurities are controlled in the drug substance and are not to be reported or included in the total degradation products for the drug product.

\(^c\)(2-Butylbenzofuran-3-yl){4-[2-(diethylamino)ethoxy]-3-iodophenyl}methanone.

\(^d\)(2-Butylbenzofuran-3-yl){4-[2-(diethylamino)ethoxy]-3,5-diiodophenyl}methanone.

**ADDITIONAL REQUIREMENTS**

**PACKAGING AND STORAGE:** Preserve in tight and light-resistant containers, and store at controlled room temperature.

**Add the following:**

**LABELING:** When more than one Dissolution test is given, the labeling states the test used only if Test 1 is not used. ▲ (RB 1-Dec-2019)

**USP REFERENCE STANDARDS (11)**

USP Amiodarone Hydrochloride RS ▲ (USP 1-Dec-2019)

**Amiodarone Hydrochloride Compounded Oral Suspension**

**DEFINITION**

Amiodarone Hydrochloride Compounded Oral Suspension contains NLT 90.0% and NMT 110.0% of the labeled amount of amiodarone hydrochloride (C\textsubscript{25}H\textsubscript{33}I\textsubscript{2}N\textsubscript{3}O\textsubscript{7} \cdot HCl).
Prepare Amiodarone Hydrochloride Compounded Oral Suspension 5 mg/mL as follows (see Pharmaceutical Compounding—Nonsterile Preparations (79S)).

Amiodarone Hydrochloride tablets$^a$ equivalent to 600 mg of amiodarone hydrochloride

| Vehicle: a 1:1 mixture of Ora-Sweet$^b$ (regular or sugar-free) and Ora-Plus$^c$, a sufficient quantity to make | 120 mL |

$^a$ Cordarone 200-mg tablets, Wyeth-Ayerst Laboratories, Philadelphia, PA.
$^b$ Paddock Laboratories, Minneapolis, MN.

Calculate the required quantity of each ingredient for the total amount to be prepared. Place the required number of Amiodarone Hydrochloride tablets in a suitable mortar and comminute to a fine powder with a pestle. Adjust the pH of the Vehicle to 6.5 ± 0.5 with a sodium bicarbonate 50-mg/mL solution prepared in Purified Water. Add the Vehicle in small portions and triturate to make a smooth paste. Add increasing volumes of the Vehicle to make an amiodarone hydrochloride liquid that is pourable. Transfer the contents of the mortar, stepwise and quantitatively, to a calibrated bottle. Add enough of the Vehicle to bring to final volume and mix well.

**ASSAY**

**PROCEDURE**

**Mobile phase:** Methanol, water, and 50 mM monobasic ammonium phosphate (0.5: 0.5: 99)

**Standard solution:** 2.5 mg/mL of USP Amiodarone Hydrochloride RS in Mobile phase

**Sample solution:** Shake thoroughly by hand each bottle of the Oral Suspension. Prepare 2.5 mg/mL of amiodarone hydrochloride from Oral Suspension and Mobile phase.

**Chromatographic system**

(See Chromatography (621), System Suitability.)

**Mode:** LC

**Detector:** UV 230 nm

**Column:** 4.6-mm × 25-cm; 10-µm packing L1

**System suitability**

**Sample:** Standard solution

**Suitability requirements**

**Relative standard deviation:** NMT 2.1% for replicate injections

**Analysis**

**Samples:** Standard solution and Sample solution

Calculate the percentage of the labeled amount of amiodarone hydrochloride (C$_{23}$H$_{32}$I$_2$NO$_7$. HCl) in the portion of Oral Suspension taken:

\[
\text{Result} = \left( \frac{r_s}{r_i} \right) \times \left( \frac{C_i}{C_u} \right) \times 100
\]

\[r_s = \text{peak response from the Sample solution} \]
\[r_i = \text{peak response from the Standard solution} \]
\[C_i = \text{concentration of USP Amiodarone Hydrochloride RS in the Standard solution (mg/mL)} \]
\[C_u = \text{nominal concentration of amiodarone hydrochloride in the Sample solution (mg/mL)} \]

**Acceptance criteria:** 90.0%–110.0%

**SPECIFIC TESTS**

**pH (791):** 5.8–6.8

**ADDITIONAL REQUIREMENTS**

**PACKAGING AND STORAGE:** Package in tight, light-resistant containers. Store in a refrigerator or at controlled room temperature.

**BEYOND-USE DATE:** NMT 90 days after the date on which it was compounded when stored in a refrigerator; NMT 30 days when stored at controlled room temperature

**LABELING:** Label it to state that it is to be well shaken before use and to state the Beyond-Use Date.

**USP REFERENCE STANDARDS** (11)

**USP Amiodarone Hydrochloride RS**

**Azeotropic Isopropyl Alcohol**

**DEFINITION**

Azeotropic Isopropyl Alcohol contains NLT 91.0% and NMT 93.0% of isopropyl alcohol, by volume, the remainder consisting of water.

**IDENTIFICATION**

**A. INFRARED ABSORPTION:** The IR absorption spectrum of a thin film of it exhibits a strong broad band at 3.0 μm; a strong region of absorption between 3.35 and 3.5 μm, with its highest peak at 3.36 μm, and others at 3.41 and 3.47 μm; many weak peaks between 3.6 and 6.0 μm, among the most noticeable being those at 3.68, 3.77, 3.97, 4.17, and 5.26 μm; a broad band at 6.2 μm; a strong region of absorption between 6.7 and 7.8 μm, the most prominent features being the peaks at 6.80, 7.09, 7.25 (the highest), 7.46, and 7.63 μm; a strong region of absorption between 8.5 and 9.2 μm, peaking at 8.6, 8.85, and 9.0 μm; and strong peaks at 10.5 and 12.3 μm.

**IMPURITIES**

**LIMIT OF NONVOLATILE RESIDUE**

Sample: 50 mL

**Analysis:** Evaporate the Sample in a tared porcelain dish on a steam bath to dryness, and heat at 105° for 1 h. The weight of the residue does not exceed 2.5 mg (0.005%).

**VOLATILE IMPURITIES**

**System suitability solution:** USP 2-Propanol System Suitability RS

**Sample solution:** Azeotropic Isopropyl Alcohol (Neat)

**Chromatographic system**

(See Chromatography (621), System Suitability.)

**Mode:** GC

**Detector:** Thermal conductivity

**Column:** 0.25-mm × 60-m, coated with a 1.4-µm film of phase G43

**Temperature**

**Injector:** 150°

**Detector:** 200°

**Column:** See Table 1.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Temperature</th>
<th>Final</th>
<th>Hold Time</th>
<th>Initial</th>
</tr>
</thead>
<tbody>
<tr>
<td>35</td>
<td>0</td>
<td>35</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>35</td>
<td>1</td>
<td>45</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>45</td>
<td>10</td>
<td>100</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

**Table 1**

Carrier gas: Helium

Linear velocity: 35 cm/s
Injection size: 1 µL
Split ratio: 10:1
Run time: 30 min
System suitability
Sample: System suitability solution
[NOTE—Approximate relative retention times for ethyl ether, acetone, isopropyl alcohol, diisopropyl ether, 1-propanol, and 2-butanol are 0.6, 0.7, 1.0, 1.1, 1.3, and 1.5, respectively.]
Suitability requirements
Resolution: NLT 1.5 between acetone and isopropyl alcohol
Signal-to-noise ratio: NLT 10 for any of the following peaks: ethyl ether, acetone, isopropyl alcohol, diisopropyl ether, 1-propanol, and 2-butanol
Tailing factor: NMT 2.0 for the isopropyl alcohol peak
Relative standard deviation: NMT 2.0% for the isopropyl alcohol peak
Analysis
Samples: Sample solution
Calculate the ratio of isopropyl alcohol (C₃H₇O) in the portion of Azeotropic Isopropyl Alcohol taken:
Result = (r₁/r₇)

\[
\begin{align*}
    r₁ & = \text{peak area for isopropyl alcohol} \\
    r₇ & = \text{sum of all of the peak areas excluding the peak for water}
\end{align*}
\]
Acceptance criteria: NLT 0.99

SPECIFIC TESTS
• SPECIFIC GRAVITY (841): 0.815–0.810, indicating 91.0%–93.0% of isopropyl alcohol (C₃H₇O) by volume
• REFRACTIVE INDEX (831): 1.376–1.378 at 20º
• ACIDITY
Sample: 50 mL
Analysis: Place the Sample in a suitable flask, and add 100 mL of carbon dioxide-free water. Add 2 drops of phenolphthalein TS, and titrate with 0.020 N sodium hydroxide to a pink color that persists for 30 s.
Acceptance criteria: NMT 0.70 mL of 0.020 N sodium hydroxide is required for neutralization.

ADDITIONAL REQUIREMENTS
• PACKAGING AND STORAGE: Preserve in tight containers, remote from heat.

Change to read:

• USP REFERENCE STANDARDS (11)
USP 2-Propanol System Suitability RS
It is a mixture of the following: ethyl ether (0.1%), acetone (0.1%), diisopropyl ether (0.1%), 1-propanol (0.1%), 2-butanol (0.1%), and isopropyl alcohol (99.5%).

Chloroquine

\[
\begin{align*}
    \text{C}_{18}\text{H}_{29}\text{ClN}_3 & = 319.87 \\
    \text{C}_{18}\text{H}_{29}\text{ClN}_3 \cdot 2\text{H}_3\text{PO}_4 & = 515.86
\end{align*}
\]
1,4-Pentanediamine, N⁴-(7-chloro-4-quinolinylo)-N³,N³-diethyl-, phosphate (1:2);
7-Chloro-4-[[4-(diethylamino)-1-methylbutyl]amino]quinoline phosphate (1:2) [50-63-5].

DEFINITION
Chloroquine Phosphate contains NLT 98.0% and NMT 102.0% of chloroquine phosphate (C_{18}H_{26}ClIN_{3} \cdot 2H_{2}PO_{4}), calculated on the dried basis.

IDENTIFICATION

Change to read:

• A. SPECTROSCOPIC IDENTIFICATION TESTS (197), Infrared Spectroscopy: 197K (CN 1-May-2020)

Change to read:

• B. SPECTROSCOPIC IDENTIFICATION TESTS (197), Ultraviolet-Visible Spectroscopy: 197U (CN 1-May-2020)

Medium: Dilute hydrochloric acid (1 in 1000)
Sample solution: 10 µg/mL
Ratio: A_{343}/A_{329}, 1.00–1.15
• C. The retention time of the major peak of the Sample solution corresponds to that of the Standard solution, as obtained in the Assay.

ASSAY

• PROCEDURE
Buffer: 1.4 g/L of anhydrous dibasic sodium phosphate in water. Adjust with 10% phosphoric acid to a pH of 3.0.
Mobile phase: 0.4% triethylamine in methanol and Buffer (70:30)
System suitability solution: 2.0 µg/mL each of USP Chloroquine Phosphate RS, USP Phenol RS, USP Hydroxychloroquine Sulfate RS, USP Chloroquine Related Compound A RS, USP Chloroquine Related Compound D RS, USP Chloroquine Related Compound E RS, and USP Chloroquine Related Compound G RS in Mobile phase
Standard solution: 0.3 mg/mL of USP Chloroquine Phosphate RS in Mobile phase. Sonicate to dissolve if necessary.
Sample solution: 0.3 mg/mL of Chloroquine Phosphate in Mobile phase. Sonicate to dissolve if necessary.

Chromatographic system
(See Chromatography (621), System Suitability.)
Mode: LC
Detector: UV 260 nm
Column: 4.6-mm × 25-cm; 5-µm packing L1
Flow rate: 1 mL/min
Injection volume: 20 µL

System suitability
Samples: System suitability solution and Standard solution
[NOTE—See Table 1 for the corresponding relative retention times.]
Suitability requirements
Resolution: NLT 2.0 between chloroquine and chloroquine related compound A, System suitability solution
Tailing factor: NMT 2.0 for chloroquine, Standard solution
Relative standard deviation: NMT 0.7% for chloroquine, Standard solution

Analysis
Samples: Standard solution and Sample solution
Calculate the percentage of chloroquine phosphate (C_{18}H_{26}ClIN_{3} \cdot 2H_{2}PO_{4}) in the portion of Chloroquine Phosphate taken:

Result = \left( \frac{r}{C} \right) \times \left( \frac{C}{C} \right) \times 100

r = peak response from the Sample solution
C = concentration of USP Chloroquine Phosphate RS in the Standard solution (mg/mL)
C = concentration of Chloroquine Phosphate in the Sample solution (mg/mL)

Acceptance criteria: 98.0%–102.0% on the dried basis

IMPURITIES

• ORGANIC IMPURITIES

Mobile phase, System suitability solution, and Chromatographic system: Proceed as directed in the Assay.

Standard solution: Use the System suitability solution.

Sample solution: 2 mg/mL of Chloroquine Phosphate in Mobile phase

System suitability
Sample: System suitability solution
[NOTE—See Table 1 for the corresponding relative retention times.]

Suitability requirements
Resolution: NLT 2.0 between chloroquine and chloroquine related compound A and NLT 2 between adjacent impurities

Tailing factor: NMT 2.0 for peaks corresponding to chloroquine phosphate, phenol, hydroxychloroquine sulfate, chloroquine related compound A, chloroquine related compound D, chloroquine related compound E, and chloroquine related compound G

Relative standard deviation: NMT 5.0% for chloroquine phosphate, phenol, hydroxychloroquine sulfate, chloroquine related compound A, chloroquine related compound D, chloroquine related compound E, and chloroquine related compound G

Analysis
Samples: Standard solution and Sample solution
Calculate the percentage of each specified impurity, other than chloroquine related compound G, in the portion of Chloroquine Phosphate taken:

Result = \left( \frac{r}{r} \right) \times \left( \frac{C}{C} \right) \times 100

r = peak response of each impurity from the Sample solution
r = peak response of the corresponding USP Reference Standard from the Standard solution
C = concentration of the corresponding USP Reference Standard in the Standard solution (mg/mL)
C = concentration of Chloroquine Phosphate in the Sample solution (mg/mL)

Calculate the percentage of chloroquine related compound G and any other unspecified impurity in the portion of Chloroquine Phosphate taken:

Result = \left( \frac{r}{r} \right) \times \left( \frac{C}{C} \right) \times 100

r = peak response of chloroquine related compound G or any other impurity from the Sample solution

Acceptance criteria: See Table 1. Disregard any peak less than 0.05%.

Table 1

<table>
<thead>
<tr>
<th>Name</th>
<th>Relative Retention Time</th>
<th>Acceptance Criteria, NMT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenol</td>
<td>0.2</td>
<td>0.1</td>
</tr>
<tr>
<td>Chloroquine related compound G</td>
<td>0.27</td>
<td>0.1</td>
</tr>
<tr>
<td>Chloroquine related compound D</td>
<td>0.42</td>
<td>0.50</td>
</tr>
<tr>
<td>Hydroxychloroquine sulfate</td>
<td>0.49</td>
<td>0.1</td>
</tr>
<tr>
<td>Chloroquine related compound A</td>
<td>0.73</td>
<td>0.1</td>
</tr>
<tr>
<td>Chloroquine phosphate</td>
<td>1.0</td>
<td>—</td>
</tr>
<tr>
<td>Chloroquine related compound E</td>
<td>1.5</td>
<td>0.1</td>
</tr>
<tr>
<td>Any other individual impurity</td>
<td>—</td>
<td>0.10</td>
</tr>
<tr>
<td>Total impurities</td>
<td>—</td>
<td>2.0</td>
</tr>
</tbody>
</table>

SPECIFIC TESTS

- **Loss on Drying (731)**
  - **Analysis:** Dry a sample at 105° for 16 h.
  - **Acceptance criteria:** NMT 2.0%

ADDITIONAL REQUIREMENTS

- **Packaging and Storage:** Preserve in well-closed containers.

- **USP Reference Standards (11)**
  - USP Chloroquine Phosphate RS
  - USP Chloroquine Related Compound A RS
  - USP Chloroquine Related Compound D RS
  - USP Chloroquine Related Compound E RS

- **USP Related Compound**
  - 5-Chloroquinoline, C<sub>8</sub>H<sub>7</sub>CIN<sub>3</sub> 291.82
  - 4,7-Dichloroquinoline, C<sub>9</sub>H<sub>5</sub>CIN<sub>4</sub> 198.05
  - Monoethyl chloroquine, C<sub>19</sub>H<sub>26</sub>CIN<sub>4</sub> 409.91
  - Chloroquine isomer, C<sub>18</sub>H<sub>17</sub>CIN<sub>3</sub>·C<sub>2</sub>H<sub>4</sub>O 343.95
  - N,N′-diethylpentane-1,4-diamine oxalate, C<sub>9</sub>H<sub>18</sub>ClIN<sub>3</sub>·H<sub>2</sub>SO<sub>4</sub> 433.95

- **System suitability**
  - **Sample:** Nominally 7.5 µg/mL of chloroquine phosphate from a filtered solution of finely powdered Tablets in water
  - **Instrumental conditions**
    - **Mode:** UV
    - **Wavelength range:** 329–343 nm
    - **Analysis**
      - **Sample solution:** Nominally 7.5 µg/mL of chloroquine phosphate from a filtered solution of finely powdered Tablets in water
      - **Analysis:** Disregard any peak less than 0.05%.

- **Chromatographic system**
  - **Mode:** LC
  - **Detector:** UV 224 nm
  - **Column:** 4.6-mm × 10-cm; 5-µm packing L1
  - **Flow rate:** 1.2 mL/min
  - **Injection volume:** 10 µL

- **System suitability**
  - **Sample:** System suitability solution
  - **[Note—]The relative retention times for chloroquine phosphate and amodiaquine hydrochloride are 1.0 and 1.3, respectively.**

- **Suitability requirements**
  - **Resolution:** NLT 1.5 between amodiaquine hydrochloride and chloroquine phosphate
  - **Tailing factor:** NMT 1.5 for the amodiaquine and chloroquine peaks
  - **Relative standard deviation:** NMT 2.0% for the amodiaquine and chloroquine peaks

Published on March 26, 2020
Chloroquine Hydrochloride Injection

C_{18}H_{26}Cl_{3}N_{2}·2HCl  392.79
1,4-Pentanediamine, N,N'-(7-chloro-4-quinolinylo)-N,N'-diethyl-, dihydrochloride;
7-(Chloro-4-[4-diethylamino]-1-methylbutyl)amino] quinoline dihydrochloride  [3545-67-3].

DEFINITION
Chloroquine Hydrochloride Injection is a sterile solution of Chloroquine in Water for Injection prepared with the aid of Hydrochloric Acid. It contains NLT 47.5 mg and NMT 52.5 mg of chloroquine hydrochloride (C_{18}H_{26}Cl_{3}N_{2}·2HCl) in each mL.

IDENTIFICATION

Change to read:

• A. SPECTROSCOPIC IDENTIFICATION TESTS (197), Ultraviolet-Visible Spectroscopy: 197Ult (CN 1-May-2020)
Sample solution: Prepare as directed in the Assay.
Standard solution: 7.5 µg/mL of USP Chloroquine Phosphate RS prepared similarly to the Sample solution.

Instrumental conditions
Mode: UV
Wavelength range: 329–343 nm

Analysis
Samples: Sample solution and Standard solution
Calculate the concentration, in mg/mL, of chloroquine hydrochloride (C_{18}H_{26}Cl_{3}N_{2}·2HCl) in the portion of Injection taken:

\[
\text{Result} = \left( \frac{A_{\text{S}}}{A_{\text{U}}} \right) \times \left( \frac{C_{\text{U}}}{C_{\text{S}}} \right) \times \left( \frac{M_{\text{r1}}}{M_{\text{r2}}} \right)
\]

A_{\text{U}} = absorbance of the Sample solution
A_{\text{S}} = absorbance of the Standard solution
C_{\text{S}} = concentration of USP Chloroquine Phosphate RS in the Standard solution (mg/mL)
D = dilution factor for the preparation of the Sample solution, 20
V = volume of the Standard solution, 1000 mL
V_{\text{i}} = volume of Injection taken for the Sample solution (mL)
M_{\text{r1}} = molecular weight of chloroquine hydrochloride, 392.79
M_{\text{r2}} = molecular weight of chloroquine phosphate, 515.86

Acceptance criteria: 47.5–52.5 mg/mL

SPECIFIC TESTS
• pH (791): 5.5–6.5

Published on March 26, 2020
Chloroquine Phosphate Compounded Oral Suspension

DEFINITION
Chloroquine Phosphate Compounded Oral Suspension contains NLT 90.0% and NMT 110.0% of the labeled amount of chloroquine phosphate (C_{18}H_{26}ClN_{5}·2H_{3}PO_{4}). Prepare Chloroquine Phosphate Compounded Oral Suspension 15 mg/mL as follows (see Pharmaceutical Compounding—Nonsterile Preparations (795)).

<table>
<thead>
<tr>
<th>Chloroquine Phosphate tablets(^a)</th>
<th>1.5 g of chloroquine phosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle: a 1:1 mixture of Ora-Sweet(^b) and Ora-Plus(^b), a sufficient quantity to make</td>
<td>100 mL</td>
</tr>
</tbody>
</table>

\(^a\) Aralen 500-mg tablets, Sanofi-Winthrop, NY.
\(^b\) Paddock Laboratories, Minneapolis, MN.

Calculate the required quantity of each ingredient for the total amount to be prepared. Place the required number of Chloroquine Phosphate tablets in a suitable mortar, and comminute to a fine powder. Add the Vehicle in small portions, and triturate to make a smooth paste. Add increasing volumes of the Vehicle to make a chloroquine phosphate liquid that is pourable. Transfer the contents of the mortar, stepwise and quantitatively, to a calibrated bottle. Add enough of the Vehicle to bring to final volume, and mix well.

ASSAY
• PROCEDURE
Buffer solution: 20 mM 1-heptanesulfonic acid adjusted to a pH of 3.4
Mobile phase: Acetonitrile and Buffer solution (34:66). Filter and degas.
Standard solution: 150 µg/mL of USP Chloroquine Phosphate RS in Mobile phase
Sample solution: Shake thoroughly by hand each bottle of Oral Suspension. Pipet 1.0 mL of the Oral Suspension into a 100-mL volumetric flask, and dilute with Mobile phase to volume.

Chromatographic system
(See Chromatography (621), System Suitability.)
Mode: LC
Detector: UV 340 nm
Column: 4.6-mm × 25-cm; 5-µm packing L1
Flow rate: 1.5 mL/min
Injection volume: 20 µL

System suitability
Sample: Standard solution
[NOTE—The retention time for chloroquine phosphate is about 9.4 min.]

Suitability requirements
Relative standard deviation: NMT 2% for replicate injections

Analysis
Samples: Standard solution and Sample solution
Calculate the percentage of the labeled amount of chloroquine phosphate (C_{18}H_{26}ClN_{5}·2H_{3}PO_{4}) in the portion of Oral Suspension taken:

\[
\text{Result} = \left(\frac{r_1}{r_2}\right) \times \left(\frac{C_i}{C_d}\right) \times 100
\]

\(r_1\) = peak response from the Sample solution
\(r_2\) = peak response from the Standard solution
\(C_i\) = concentration of USP Chloroquine Phosphate RS in the Standard solution (µg/mL)
\(C_d\) = nominal concentration of chloroquine phosphate in the Sample solution (µg/mL)

Acceptance criteria: 90.0%–110.0%

SPECIFIC TESTS
• pH (791): 4.0–5.0

ADDITIONAL REQUIREMENTS
• PACKAGING AND STORAGE: Package in tight, light-resistant containers. Store at controlled room temperature or in a refrigerator.
• BEYOND-USE DATE: NMT 60 days after the date on which it was compounded when stored in a refrigerator or at controlled room temperature
• LABELING: Label it to indicate that it is to be well shaken before use, and to state the Beyond-Use Date.
• USP REFERENCE STANDARDS (11)
USP Chloroquine Phosphate RS

Chlorpromazine

\[
C_{17}H_{19}ClN_{5}S
\]
318.86
10H-Phenothiazine-10-propanamine, 2-chloro-N,N-dimethyl-
2-Chloro-10-[3-(dimethylamino)propyl]phenothiazine [50-53-3].

» Chlorpromazine contains not less than 98.0 percent and not more than 101.0 percent of C_{17}H_{19}ClN_{5}S, calculated on the dried basis.

Packaging and storage—Preserve in tight, light-resistant containers.

USP Reference standards (11)—
USP Chlorpromazine Hydrochloride RS
[NOTE—Throughout the following procedures, protect test or assay specimens, the Reference Standard, and solutions containing them by conducting the procedures without delay, under subdued light, or using low-actinic glassware.]

Identification—
A: The IR absorption spectrum of a 1 in 100 solution in carbon disulfide, in a 1.0-mm cell between 7 µm and 15 µm, exhibits maxima only at the same wavelengths as that of a solution prepared by dissolving 55 mg of USP Chlorpromazine Hydrochloride RS in 3 mL of 1 N sodium hydroxide and

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extracting the resulting solution with 5.0 mL of carbon
disulfide.

B: The principal spot found in the test for Other alkylated phenothiazines corresponds in R_f to the spot from the Standard solution.

Loss on drying (731)—Dry it in vacuum at room temperature for 3 hours: it loses not more than 1.0% of its weight.

Other alkylated phenothiazines—Dissolve 45.0 mg in 10 mL of methanol. Dissolve a suitable quantity of USP Chlorpromazine Hydrochloride RS in methanol to obtain a concentration of 5 mg per mL (Standard solution), and dilute it quantitatively and stepwise with methanol to obtain a concentration of 25 µg per mL (Diluted standard solution). Apply separately 10 µL of each of the three solutions to the starting line of a thin-layer chromatographic plate coated with chromatographic silica gel mixture. Develop the chromatogram, using as the solvent system a freshly prepared mixture of equal volumes of ether and ethyl acetate saturated with ammonium hydroxide, until the solvent front has moved about 10 cm from the origin. Remove the plate from the chamber, and air-dry for 20 minutes. View under short-wavelength UV light: the area and intensity of any spot, other than the principal spot, from the solution of Chlorpromazine are not greater than those of the spot from the Diluted standard solution (0.5%).

Assay—Place about 750 mg of Chlorpromazine, accurately weighed, in a 250-mL conical flask, and dissolve in 25 mL of glacial acetic acid, warming gently on a steam bath to effect solution. Cool, add crystal violet TS, and titrate with 0.1 N perchloric acid VS to a blue endpoint. Perform a blank determination, and make any necessary correction. Each mL of 0.1 N perchloric acid is equivalent to 31.89 mg of \( C_{17}H_{19}ClN_2S \cdot HCl \).

Chlorpromazine Hydrochloride Tablets

Chlorpromazine Hydrochloride Tablets contain not less than 95.0 percent and not more than 105.0 percent of the labeled amount of \( C_{17}H_{19}ClN_2S \cdot HCl \).

Packaging and storage—Preserve in well-closed, light-resistant containers.

USP Reference standards (11)—USP Chlorpromazine Hydrochloride RS

Identification—

A: Tablets respond to Identification test B under Chlorpromazine Hydrochloride.

B: Digest a quantity of powdered Tablets, equivalent to about 25 mg of chlorpromazine hydrochloride, with 25 mL of water, and filter: the solution so obtained responds to Identification test C under Chlorpromazine Hydrochloride Hydrochloride.

Dissolution (711)—

Medium: 0.1 N hydrochloric acid; 900 mL.
Apparatus 1: 50 rpm.
Time: 30 minutes.

Procedure—Determine the amount of \( C_{17}H_{19}ClN_2S \cdot HCl \) dissolved from UV absorbances at the wavelength of maximum absorbance at about 254 nm of filtered portions of the solution under test, suitably diluted with Dissolution Medium, in comparison with a Standard solution having a known concentration of USP Chlorpromazine Hydrochloride RS in the same medium.

Tolerances—Not less than 80% (Q) of the labeled amount of \( C_{17}H_{19}ClN_2S \cdot HCl \) is dissolved in 30 minutes.

Uniformity of dosage units (90S): meet the requirements.

Other alkylated phenothiazines—Transfer a portion of finely powdered Tablets, equivalent to 50 mg of chlorpromazine hydrochloride, to a stopped centrifuge tube, add 10 mL of methanol, shake vigorously, and centrifuge (remove any sugar coating by prior washing with water).
Proceed as directed in the test for Other alkylation phenothiazines under Chlorpromazine, beginning with "Dissolve a suitable quantity of USP Chlorpromazine Hydrochloride RS." The area and intensity of any spot, other than the principal spot, from the solution from the Tablets are not greater than those of the spot from the Diluted standard solution (0.5%).

**Assay**—Weigh and finely powder, not fewer than 20 Tablets. Transfer an accurately weighed portion of the powder, equivalent to about 100 mg of chlorpromazine hydrochloride, to a 500-mL volumetric flask. Add about 200 mL of water and 5 mL of hydrochloric acid, insert the stopper, and shake for about 10 minutes. Dilute with water to volume, and mix. Filter a portion of the solution, discarding the first 50 mL of the filtrate. Treat 10.0 mL of the filtrate as directed in the Assay under Chlorpromazine Hydrochloride Injection, beginning with "Pipet 10 mL of the solution." Calculate the quantity, in µg per mL, of USP Chlorpromazine Hydrochloride RS in the Standard solution, and the parenthetic expressions are the differences in the absorbances of the two solutions at the wavelengths indicated by the subscripts, for the solution from the Tablets (U) and the Standard solution (S), respectively.

\[ 12.5C(A_{254} - A_{277})/V(A_{254} - A_{277}) \]

in which C is the concentration, in µg per mL, of USP Chlorpromazine Hydrochloride RS in the Standard solution, and the parenthetic expressions are the differences in the absorbances of the two solutions at the wavelengths indicated by the subscripts, for the solution from the Tablets (U) and the Standard solution (S), respectively.

---

**Chlorpromazine Hydrochloride Syrup**

» Chlorpromazine Hydrochloride Syrup contains, in each 100 mL, not less than 190 mg and not more than 210 mg of C₁₇H₁₅ClN₂S · HCl.

**Packaging and storage**—Preserve in tight, light-resistant containers.

**USP Reference standards** (11)—

USP Chlorpromazine Hydrochloride RS

[Note—Throughout the following procedures, protect test or assay specimens, the Reference Standard, and solutions containing them by conducting the procedures without delay, under subdued light, or using low-actinic glassware.]

**Identification**—

A: Transfer a volume of it, equivalent to about 20 mg of chlorpromazine hydrochloride, to a 125-mL separator. Add 10 mL of water, 2 mL of sodium hydroxide solution (1 in 2), and mix. Extract with three 30-mL portions of ether. Filter the combined ether extracts through anhydrous sodium sulfate. With the aid of a stream of nitrogen evaporate the ether to about 5 mL. Quantitatively transfer the solution to a 40-mL centrifuge tube. Evaporate with a stream of nitrogen to dryness. Dissolve the residue in 1.0 mL of methanol and spray with iodoplatinate reagent prepared by dissolving 100 mg of platinic chloride in 10 mL of 0.1 N hydrochloric acid, adding 25 mL of potassium iodide solution (1 in 25), 0.5 mL of formic acid, and diluting with water to 100 mL: the Rₐ value of the principal spot from the test solution corresponds to that obtained from the Standard solution.

B: Dilute a portion of the Syrup with an equal volume of water: the resulting solution responds to the tests for Chloride (191).

**Limit of chlorpromazine sulfoxide**—

Chlorpromazine sulfoxide standard solution—Transfer 5 mL of a solution in dilute hydrochloric acid (1 in 100) of USP Chlorpromazine Hydrochloride RS containing 10.6 mg per mL to a 50-mL volumetric flask. Add 2 mL of 30% hydrogen peroxide and heat at 60° for 10 minutes. Cool, dilute with 1 M sodium bisulfite to volume, and mix. Transfer 10.0 mL to a 60-mL separator, add 2 mL of sodium hydroxide solution (1 in 2), and mix. Extract with three 30-mL portions of ether. Filter the combined ether extracts through anhydrous sodium sulfate into a 250-mL conical flask. Cautiously evaporate the extracts to dryness. Dissolve the residue in 10.0 mL of methanol, and filter if necessary. Each mL of this solution contains 1 mg of chlorpromazine sulfoxide.

**Procedure**—Transfer an accurately measured volume of the Syrup, equivalent to about 20 mg of chlorpromazine hydrochloride, to a 125-mL separator. Add 10 mL of water and 2 mL of sodium hydroxide solution (1 in 2), and mix. Extract with three 30-mL portions of ether. Filter the combined ether extracts through anhydrous sodium sulfate. With the aid of a stream of nitrogen evaporate the ether to about 5 mL. Quantitatively transfer the solution to a 40-mL centrifuge tube. Evaporate with a stream of nitrogen and mild heat to dryness. Dissolve the residue in 1.0 mL of methanol to obtain the Test solution. Separately apply 15 µL of this Test solution and 15 µL of a Chlorpromazine sulfoxide standard solution to a thin-layer chromatographic plate (see Chromatography (621)) coated with a 0.25-mm layer of chromographic silica gel. Develop the chromatogram in a chamber containing a freshly prepared mixture of ethyl acetate that has been saturated with ammonium hydroxide, ether, and methanol (7:5:2.5:20) until the solvent front has moved about three-fourths of the length of the plate. Remove the plate from the chamber, air-dry, and spray with iodoplatinate reagent prepared by dissolving 100 mg of platinic chloride in 10 mL of 0.1 N hydrochloric acid, adding 25 mL of potassium iodide solution (1 in 25), and 0.5 mL of formic acid, and diluting with water to 100 mL: the Rₐ value of the principal spot on the chromatogram from the Test solution may exhibit a secondary intensity are not greater than, those of the spot from the Syrup (U) and the Standard solution (S), respectively.

\[ 1.25C(A_{254} - A_{277})/V(A_{254} - A_{277}) \]

in which C is the concentration, in µg per mL, of USP Chlorpromazine Hydrochloride RS in the Standard solution; V is the volume, in mL, of Syrup taken; and the parenthetic expressions are the differences in the absorbances of the two solutions at the wavelengths indicated by the subscripts, for the solution from the Syrup (U) and the Standard solution (S), respectively.
Chlorpromazine Hydrochloride Injection

> Chlorpromazine Hydrochloride Injection is a sterile solution of Chlorpromazine Hydrochloride in Water for Injection. It contains, in each mL, not less than 23.75 mg and not more than 26.25 mg of C₁₇H₁₉ClN₄S · HCl.

Packaging and storage—Preserve in single-dose or in multiple-dose containers, preferably of Type I glass, protected from light.

USP Reference standards (11)—

USP Chlorpromazine Hydrochloride RS

[Note—Throughout the following procedures, protect test or assay specimens, the Reference Standard, and solutions containing them by conducting the procedures without delay, under subdued light, or using low-actinic glassware.]

Identification—

A: Transfer a volume of Injection, equivalent to about 25 mg of chlorpromazine hydrochloride, to a 10-mL volumetric flask, dilute with methanol to volume, and mix (test solution). Dissolve a suitable quantity of USP Chlorpromazine Hydrochloride RS in dilute methanol (9 in 10) to obtain a Standard solution having a known concentration of 2.5 mg per mL. Apply separately 5-µL portions of each of the two solutions to the starting line of a thin-layer chromatographic plate (see Chromatography (621)) coated with chromatographic silica gel mixture. Develop the chromatogram in a solvent system consisting of a freshly prepared mixture of equal volumes of ether and ethyl acetate saturated with ammonium hydroxide until the solvent front has moved about 10 cm from the origin. Remove the plate from the developing chamber, air-dry for 20 minutes, then view under short-wavelength UV light: the area and intensity of the principal spot, are not greater than those of the spot from the test solution.

B: It responds to the tests for Chloride (191).

Bacterial Endotoxins Test (85) —It contains not more than 6.9 USP Endotoxin Units per mg of chlorpromazine hydrochloride.

pH (791): between 3.4 and 5.4.

Limit of chlorpromazine sulfoxide—[Note—Conduct this test without exposure to daylight, and with the minimum necessary exposure to artificial light.]

Test preparation—Pipet 4 mL of the test solution prepared with methanol as directed in Identification test A into a 10-mL volumetric flask, dilute with methanol to volume, and mix.

Standard preparation—Dissolve a suitable quantity of USP Chlorpromazine Hydrochloride RS in methanol to obtain a solution having a concentration of 50 µg per mL.

Procedure—Apply separate 10-µL portions of the Standard preparation and the Test preparation to the starting line of a thin-layer chromatographic plate coated with a 0.25-mm layer of chromatographic silica gel mixture. Dry the applied solutions with the aid of a stream of nitrogen. Develop the chromatogram, using as the solvent system a freshly prepared mixture of equal volumes of ether and ethyl acetate saturated with ammonium hydroxide, until the solvent front has moved about 13 cm from the origin. Remove the plate from the chamber, and air-dry for 30 minutes. Examine under short-wavelength UV light: the area and intensity of the only other spot in the test specimen chromatogram, other than the principal spot, are not greater than those of the spot from the Standard preparation (5.0%).

Other requirements—It meets the requirements under Injections and Implanted Drug Products (1).

Assay—Transfer an accurately measured volume of Injection, equivalent to about 100 mg of chlorpromazine hydrochloride, to a 500-mL volumetric flask, add 0.1 N hydrochloric acid to volume, and mix. Pipet 10 mL of the solution into a 250-mL separator, add about 20 mL of water, render alkaline with ammonium hydroxide, and extract with four 25-mL portions of ether. Extract the combined ether extracts with four 25-mL portions of 0.1 N hydrochloric acid, collecting the aqueous extracts in a 250-mL volumetric flask. Aerate to remove residual ether, add 0.1 N hydrochloric acid to volume, and mix. Dissolve a suitable quantity, accurately weighed, of USP Chlorpromazine Hydrochloride RS in 0.1 N hydrochloric acid, and dilute quantitatively and stepwise with the same acid to obtain a Standard solution having a known concentration of about 8 µg per mL. Concomitantly determine the absorbances of both solutions in 1-cm cells at the wavelength of maximum absorbance at about 254 nm and at 277 nm, with a suitable spectrophotometer, using 0.1 N hydrochloric acid as the blank. Calculate the quantity, in mg, of C₁₇H₁₉ClN₄S · HCl in each mL of the Injection taken by the formula:

\[
12.5(C(A_{254} - A_{277}) / V(A_{254} - A_{277}) )^{-1} \times 1000
\]

in which C is the concentration, in µg per mL, of USP Chlorpromazine Hydrochloride RS in the Standard solution, V is the volume, in mL, of Injection taken, and the parenthetic expressions are the differences in the absorbances of the two solutions at the wavelengths indicated by the subscripts, for the solution from the Injection (U) and the Standard solution (S), respectively.

Chlorpromazine Hydrochloride Oral Concentrate

> Chlorpromazine Hydrochloride Oral Concentrate contains not less than 90.0 percent and not more than 110.0 percent of the labeled amount of C₁₇H₁₉ClN₄S · HCl.

Packaging and storage—Preserve in tight, light-resistant containers.

Labeling—Label it to indicate that it must be diluted prior to administration.

USP Reference standards (11)—

USP Chlorpromazine Hydrochloride RS

[Note—Throughout the following procedures, protect test or assay specimens, the Reference Standard, and solutions containing them by conducting the procedures without delay, under subdued light, or using low-actinic glassware.]

Identification—

A: It responds to Identification test A under Chlorpromazine Hydrochloride Syrup.

B: Dilute a portion of the Oral Concentrate with an equal volume of water: the resulting solution responds to the tests for Chloride (191).

Microbial enumeration tests (61) and Tests for specified microorganisms (62)—It meets the requirements of the tests for the absence of Escherichia coli.

pH (791): between 2.3 and 4.1.

Limit of chlorpromazine sulfoxide—Proceed as directed in the test for Chlorpromazine sulfoxide under Chlorpromazine Hydrochloride Syrup.
**Assay**—Transfer an accurately measured volume of Oral Concentrate, previously diluted if necessary, equivalent to about 10 mg of chlorpromazine hydrochloride, to a 50-mL volumetric flask, add 0.1 N hydrochloric acid to volume, and mix. Proceed as directed in the Assay under Chlorpromazine Hydrochloride Injection, beginning with “Pipet 10 mL of the solution.” Calculate the quantity, in mg, of Chlorpromazine Hydrochloride Injection in each mL of the Oral Concentrate taken by the formula:

\[
1.25C(A_{254} - A_{277})/V(A_{254} - A_{277})S
\]

in which C is the concentration, in \( \mu g \) per mL, of USP Chlorpromazine Hydrochloride RS in the Standard solution; V is the volume, in mL, of Oral Concentrate taken; and the parenthetic expressions are the differences in the absorbances of the two solutions at the wavelengths indicated by the subscripts, for the solution from the Oral Concentrate (U) and the Standard solution (S), respectively.

**Cyclosporine**

Cyclosporine contains NLT 97.0% and NMT 101.5% of cyclosporine A (C_{37}H_{111}N_{11}O_{12}), calculated on the dried basis.

**IDENTIFICATION**

- A. The retention time of the major peak of the Sample solution corresponds to that of the Standard solution, as obtained in the Assay.

**ASSAY**

- **PROCEDURE**
  - Mobile phase: Acetonitrile, tert-butyl methyl ether, water, and phosphoric acid (430:50:520:1)
  - Diluent: Acetonitrile and water (1:1)
  - System suitability solution: 1.25 mg/mL of USP Cyclosporine Resolution Mixture RS in Diluent
  - Standard solution: 1.25 mg/mL of USP Cyclosporine RS in Diluent
  - Sample solution: 1.25 mg/mL of Cyclosporine in Diluent Chromatographic system
    (See Chromatography (621), System Suitability.)
  - Mode: LC
  - Detector: UV 210 nm

**Column**: 4-mm × 25-cm; 3- to 5-µm packing L1; with 0.25-mm × 1-m stainless steel tubing connected to the column inlet

**Column temperature**: 80°. The tubing and column are maintained at 80°, to ensure that the Mobile phase entering the column is heated to 80°.

**Flow rate**: 1.2 mL/min

**Injection volume**: 20 µL

**System suitability**

- **Samples**: System suitability solution and Standard solution
  - **Samples**: Standard solution and Sample solution

Calculate the percentage of cyclosporine (C_{37}H_{111}N_{11}O_{12}) in the portion of Cyclosporine taken:

\[
\text{Result} = (r_u/r_s) \times (C_u/C_s) \times P \times 100
\]

- \( r_u \) = peak area of cyclosporine from the Sample solution
- \( r_s \) = peak area of cyclosporine from the Standard solution
- \( C_u \) = concentration of the Standard solution (mg/mL)
- \( C_s \) = concentration of the Sample solution (mg/mL)
- \( P \) = potency of cyclosporine in USP Cyclosporine RS (mg/mg)

**Acceptance criteria**: 97.0%–101.5% on the dried basis

**IMPURITIES**

- **ORGANIC IMPURITIES**
  - Mobile phase, Diluent, System suitability solution, Sample solution, and Chromatographic system:
    Proceed as directed in the Assay.
  - **Standard solution**: 0.01 mg/mL of USP Cyclosporine RS in Diluent

**Suitability requirements**

- **Resolution**:
  - NLT 1.0 between cyclosporine U and cyclosporine, System suitability solution

**Relative standard deviation**: NMT 10.0%, Standard solution

**Analysis**

- **Samples**: Standard solution and Sample solution

Calculate the percentage of each impurity in the portion of Cyclosporine taken:

\[
\text{Result} = (r_u/r_s) \times (C_u/C_s) \times P \times 100
\]

- \( r_u \) = peak area of an individual impurity from the Sample solution
- \( r_s \) = peak area of cyclosporine from the Standard solution
- \( C_u \) = concentration of the Sample solution (mg/mL)
- \( C_s \) = concentration of the Sample solution (mg/mL)
- \( P \) = potency of cyclosporine in USP Cyclosporine RS (mg/mg)

**Acceptance criteria**

- Reporting tolerance is 0.05%.
- Any individual impurity: NMT 0.7%
Cyclosporine Capsules

Cyclosporine Capsules contain not less than 90.0 percent and not more than 110.0 percent of the labeled amount of cyclosporine (C_{62}H_{111}N_{11}O_{12}).

Packaging and storage—Preserve in tight containers, and store at controlled room temperature.

Identification—The retention time of the major peak in the chromatogram of the Assay preparation corresponds to that in the chromatogram of the Standard preparation, as obtained in the Assay.

Dissolution (711)—

W I R E D  C A P S U L E S  C O N T A I N  L I Q U I D —

Medium: water; 500 mL.

Apparatus 2: 50 rpm.

Time: 15 minutes.

Procedure—Place 1 Capsule in each vessel, and allow the Capsule to sink to the bottom of the vessel before starting rotation of the blade. Observe the Capsules, and record the time taken for each Capsule shell to rupture.

Tolerances—The requirements are met if all of the Capsules tested rupture in not more than 15 minutes. If 1 or 2 of the Capsules rupture in more than 15 but not more than 30 minutes, repeat the test on 12 additional Capsules. Not more than 2 of the total of 18 Capsules tested rupture in more than 15 but not more than 30 minutes.

W I R E D  C A P S U L E S  C O N T A I N  P O W D E R —

Medium: 0.1 N hydrochloric acid containing 0.5% of sodium lauryl sulfate; 1000 mL.

Apparatus 1: 150 rpm.

Time: 90 minutes.

Determine the amount of C_{62}H_{111}N_{11}O_{12} dissolved by employing the following method.

Mobile phase—Prepare a filtered and degassed mixture of acetonitrile, water, methanol, and phosphoric acid (900: 450: 50: 0.5). Make adjustments if necessary (see System Suitability under Chromatography (621)).

Standard solution—Quantitatively dissolve an accurately weighed quantity of USP Cyclosporine RS in Dissolution Medium to obtain a solution having a known concentration of about 0.001 mg per mL, L being the labeled quantity, in mg, of cyclosporine in each Capsule. Transfer 25.0 mL of this solution to a 50-mL volumetric flask, dilute with acetonitrile to volume, and mix. This solution contains about 0.0005 L mg of USP Cyclosporine RS per mL.

Test solution—Filter a portion of the solution under test. Transfer 5.0 mL of the filtrate to a 10-mL volumetric flask, dilute with acetonitrile to volume, and mix.

Chromatographic system (see Chromatography (621))—The liquid chromatograph is equipped with a 210-nm detector and a 4.6-mm × 25-cm column that contains packing L1 and is maintained at a constant temperature of about 80°C. The flow rate is about 2 mL per minute. Chromatograph the Standard solution, and record the peak areas as directed for Procedure: the column efficiency is not less than 700 theoretical plates; and the relative standard deviation for replicate injections is not more than 2.0%.

Procedure—Separately inject equal volumes (about 10 µL) of the solution estimated to contain 0.1 mg of cyclosporine per mL, or 40 µL of the solution estimated to contain 0.025 mg of cyclosporine per mL of the Standard solution and the Test solution into the chromatograph, record the chromatograms, and measure the peak areas for the major peaks. Calculate the quantity, in mg, of C_{62}H_{111}N_{11}O_{12} dissolved by the formula:

\[
2000C(f_{r_2}/f_{r_1})
\]

in which C is the concentration, in mg per mL, of USP Cyclosporine RS in the Standard solution; and f_{r_2} and f_{r_1} are the cyclosporine peak areas obtained from the Test solution and the Standard solution, respectively.

Tolerances—Not less than 80% (Q) of the labeled amount of C_{62}H_{111}N_{11}O_{12} dissolved in 90 minutes.

Uniformity of dosage units (905): meet the requirements.

Water Determination, Method I (921) —For Capsules that contain powder, not more than 3.5% is found, using finely ground Capsule contents.

Assay—

W I R E D  C A P S U L E S  C O N T A I N  L I Q U I D —

Mobile phase and Chromatographic system—Proceed as directed in the Assay under Cyclosporine Capsules.

Standard preparation—Dissolve an accurately weighed quantity of USP Cyclosporine RS in dehydrated alcohol to obtain a solution having a known concentration of about 1 mg per mL. Use this solution promptly after preparation.

Assay preparation—Using a sharp blade, carefully cut open not fewer than 20 Capsules, and with the aid of dehydrated alcohol transfer the contents of the Capsules to a suitable volumetric flask. Wash the blade with dehydrated alcohol, and transfer the washings to the volumetric flask. Dilute the contents of the volumetric flask with dehydrated alcohol to volume, and mix. Quantitatively dilute an accurately measured volume of this solution with dehydrated alcohol to obtain a solution having a concentration of about 1 mg of cyclosporine per mL.

Procedure—Separately inject equal volumes (about 20 µL) of the Standard preparation and the Assay preparation into the chromatograph, record the chromatograms, and measure the peak areas for the major peaks. Calculate the quantity, in mg, of cyclosporine (C_{62}H_{111}N_{11}O_{12}) in each Capsule taken by the formula:

\[
(L/D)(CP/1000)(r_{r_2}/r_{r_1})
\]

in which L is the labeled quantity, in mg, of cyclosporine in each Capsule taken; D is the concentration, in mg per mL, of the Assay preparation, based on the labeled quantity of cyclosporine in the Capsules taken and the extent of dilution;
C is the concentration, in mg per mL, of USP Cyclosporine RS in the Standard preparation; \( P \) is the purity, in \( \mu \)g per mg, of USP Cyclosporine RS; and \( t_r \) and \( r \) are the peak areas obtained from the Assay preparation and the Standard preparation, respectively.

**WHERE CAPSULES CONTAIN POWDER—**

**Mobile phase**—Prepare a filtered and degassed mixture of acetonitrile, water, methanol, and phosphoric acid (605: 400: 50: 0.5). Make adjustments if necessary (see System Suitability under Chromatography (621)).

**Diluting solvent**—Prepare a mixture of acetonitrile, tetrahydrofuran, and dehydrated alcohol (9:5:4).

**Standard preparation**—Transfer about 25 mg of USP Cyclosporine RS, accurately weighed, to a 25-mL volumetric flask. Add 2.5 mL of water, and sonicate for 10 minutes. Add about 10 mL of Diluting solvent, sonicate for 5 minutes, dilute with Diluting solvent to volume, and mix.

**Assay stock preparation**—Transfer the contents of 20 Capsules to a volumetric flask of such capacity, \( V \), in mL, to make a final concentration of 10 mg of cyclosporine per mL. Add 0.1 V mL of water to the flask, and sonicate for 10 minutes. Add 0.4 V mL of Diluting solvent to the flask, and sonicate for 5 minutes. Dilute with Diluting solvent to volume, and mix.

**Assay preparation**—Transfer 5.0 mL of Assay stock preparation to a 50-mL volumetric flask, add 5 mL of water, dilute with Diluting solvent to volume, and mix.

**Chromatographic system** (see Chromatography (621))—The liquid chromatograph is equipped with a 210-nm detector and a 4.6-mm × 25-cm column that contains packing L13 and packing L16.

Chromatography—Transfer the contents of 20 Capsules into the chromatograph, record the chromatograms, and measure the areas for the major peaks. Calculate the quantity, in mg, of cyclosporine \((C_{46}H_{111}N_{11}O_{12})\) in each Capsule taken by the formula:

\[
10CV(t_r/t)
\]

in which \( C \) is the concentration, in mg per mL, of USP Cyclosporine RS in the Standard preparation; \( V \) is the volume, in mL, of the volumetric flask used to prepare the Assay stock preparation; and \( t_r \) and \( t \) are the cyclosporine peak areas obtained from the Assay preparation and the Standard preparation, respectively.

**Sample solution**: Nominally 0.5 mg/mL of cyclosporine from Injection in methanol

**Chromatographic system**

(See Chromatography (621), Thin-Layer Chromatography.)

**Adsorbent**: 0.25-mm layer of chromatographic silica gel mixture

**Application volume**: 10 \( \mu \)L

**Developing solvent system 1**: Ethyl ether

**Developing solvent system 2**: Ethyl acetate, methyl ethyl ketone, water, and formic acid (60:40:2:1)

**Spray reagent 1**: Mix 5 mL of Solution A with 5 mL of Solution B and 20 mL of glacial acetic acid, and dilute with water to 100 mL. Prepare freshly.

**Spray reagent 2**: Hydrogen peroxide TS

**Analysis**

**Samples**: Standard solution and Sample solution

Apply the Standard solution and the Sample solution to the plate. Allow the spots to dry in a current of air, place the plate in a suitable chromatographic chamber, and develop the chromatogram, using Developing solvent system 1, until the solvent front has moved about three-fourths of the length of the plate. Remove the plate from the chamber, mark the solvent front, and allow it to dry. Place the plate in a second chromatographic chamber, and develop the chromatogram in Developing solvent system 2 until the solvent front has moved about three-fourths of the length of the plate. Remove the plate from the chamber, and allow it to dry. Spray the plate with Spray reagent 1. Immediately again spray the plate with Spray reagent 2. Cyclosporine appears as a brown spot having an \( R_f \) value of about 0.45.

**Acceptance criteria**: The \( R_f \) value of the principal spot of the Sample solution corresponds to that of the Standard solution. Disregard any spots at the origin.

**B.** The retention time of the major peak of the Sample solution corresponds to that of the Standard solution, as obtained in the Assay.

**ASSAY**

**PROCEDURE**

**Mobile phase**: Acetonitrile, methanol, water, and phosphoric acid (550: 50: 400: 0.5)

**Standard solution**: 0.5 mg/mL of USP Cyclosporine RS in methanol. Use this solution promptly after preparation.

**Sample solution 1** (where it is represented as being in a single-dose container): Nominally 0.5 mg/mL of cyclosporine from Injection in methanol, prepared as follows. Using a suitable hypodermic needle and syringe, withdraw all of the withdrawable contents from 1 container of Injection, and dilute with methanol. Use this solution promptly after preparation.

**Sample solution 2** (where the label states the quantity of cyclosporine in a given volume): Nominally 0.5 mg/mL of cyclosporine from Injection in methanol, prepared as follows. Dilute a suitable aliquot of Injection with methanol. Use this solution promptly after preparation.

**Chromatographic system**

(See Chromatography (621), System Suitability.)

**Mode**: LC

**Detector**: UV 210 nm

**Capacity factor**: NLT 3–NMT 10

**Column efficiency**: NLT 700 theoretical plates

**System suitability**

**Sample**: Standard solution

**Capacity factor**: NLT 3–NMT 10

**Column efficiency**: NLT 700 theoretical plates

**Published on March 26, 2020**
Tailing factor: NMT 1.5
Relative standard deviation: NMT 1.5%

**Analysis**

Samples: Standard solution and Sample solution 1 or Sample solution 2

Calculate the percentage of the labeled amount of cyclosporine (C₆₂H₁₁₁N₁₈O₁₂) in the portion of Injection taken:

\[
\text{Result} = \left( \frac{R_\text{U}}{R_\text{S}} \right) \times \left( \frac{C_\text{U}}{C_\text{S}} \right) \times 100
\]

\( R_\text{U} \) = peak response from Sample solution 1 or Sample solution 2
\( R_\text{S} \) = peak response from the Standard solution
\( C_\text{S} \) = concentration of alcohol in the Standard solution (mg/mL)
\( C_\text{U} \) = nominal concentration of Sample solution 1 or Sample solution 2 (mg/mL)

Acceptance criteria: 90.0%–110.0%

**SPECIFIC TESTS**

- **BACTERIAL ENDOTOXINS TEST (85)**
  Sample solution: Make a 1:10 dilution of the Injection with Water for Injection.
  Analysis: Add 0.1 mL of Sample solution and 0.1 mL of appropriately constituted LAL reagent to a suitable pyrogen-free test tube. Mix on a vortex mixer for about 5 s. Acceptance criteria: NMT 0.84 USP Endotoxin Unit/mg of cyclosporine

- **STERILITY TESTS (71)**: Meets the requirements

**ADDITIONAL REQUIREMENTS**

- **PACKAGING AND STORAGE:** Preserve in single-dose or multiple-dose containers.
- **LABELING:** Label it to indicate that it is to be diluted with a suitable parenteral vehicle before intravenous infusion.
- **USP REFERENCE STANDARDS (11)**
  USP Cyclosporine RS

---

**Cyclosporine Oral Solution**

**DEFINITION**

Cyclosporine Oral Solution is a solution of Cyclosporine in a suitable vehicle. It contains NLT 90.0% and NMT 110.0% of the labeled amount of cyclosporine (C₆₂H₁₁₁N₁₈O₁₂).

**IDENTIFICATION**

- **A. THIN-LAYER CHROMATOGRAPHY**
  Solution A: 17 mg/mL of bismuth subnitrate in 20% acetic acid
  Solution B: 400 mg/mL of potassium iodide
  Diluent: Methanol and chloroform (4:1)
  Standard solution: 1 mg/mL of USP Cyclosporine RS in Diluent
  Sample solution: Nominally 1 mg/mL of cyclosporine from Oral Solution in Diluent

**CHROMATOGRAPHIC SYSTEM**

(See Chromatography (621), Thin-Layer Chromatography.)

**Adsorbent:** 0.25-mm layer of chromaticographic silica gel mixture

**Application volume:** 10 μL

**Developing solvent system 1:** Ethyl ether

**Developing solvent system 2:** Ethyl acetate, methyl ethyl ketone, water, and formic acid (60:40:2:1)

**Spray reagent 1:** Mix 5 mL of Solution A with 5 mL of Solution B and 20 mL of glacial acetic acid, and dilute with water to 100 mL. Prepare freshly.

**Spray reagent 2:** Hydrogen peroxide TS

**Analysis**

Samples: Standard solution and Sample solution

Apply the Standard solution and the Sample solution to the plate. Allow the spots to dry in a current of air, place the plate in a suitable chromatographic chamber, and develop the chromatogram, using Developing solvent system 1, until the solvent front has moved about three-fourths of the length of the plate. Remove the plate from the chamber, mark the solvent front, and allow it to dry. Place the plate in a second chromatographic chamber

---

Table 1

<table>
<thead>
<tr>
<th>Initial Temperature (°C)</th>
<th>Temperature Ramp (°C/min)</th>
<th>Final Temperature (°C)</th>
<th>Hold Time at Final Temperature (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>145</td>
<td>0</td>
<td>145</td>
<td>8</td>
</tr>
<tr>
<td>145</td>
<td>32</td>
<td>270</td>
<td>0</td>
</tr>
</tbody>
</table>

Carrier gas: Nitrogen
Flow rate: 35 mL/min
Injection volume: 1 μL. [NOTE—Make adjustments, if necessary, to obtain satisfactory chromatography.]

**System suitability**

Sample: Standard solution
Suitability requirements: [NOTE—The elution order is alcohol, n-propyl alcohol, and butyl alcohol.]
Relative standard deviation: NMT 2.0%

**Analysis**

Samples: Standard solution and Sample solution

Calculate the percentage of the labeled amount of alcohol (C₆₂H₁₁₁OH) in the portion of Injection taken:

\[
\text{Result} = \left( \frac{R_\text{U}}{R_\text{S}} \right) \times \left( \frac{C_\text{U}}{C_\text{S}} \right) \times 100
\]

\( R_\text{U} \) = peak area ratio of alcohol to n-propyl alcohol from the Sample solution
\( R_\text{S} \) = peak area ratio of alcohol to n-propyl alcohol from the Standard solution
\( C_\text{S} \) = concentration of alcohol in the Standard solution (mg/mL)
\( C_\text{U} \) = concentration of the Sample solution (mg/mL)

Acceptance criteria: 80.0%–120.0% of the labeled amount of alcohol
The retention time of the major peak of the Sample solution corresponds to that of the Standard solution, as obtained in the Assay.

ASSAY

PROCEDURE

Mobile phase: Acetonitrile, methanol, water, and phosphoric acid (350: 50: 400: 0.5)

Diluent: Methanol and chloroform (4:1)

Standard solution: 1 mg/mL of USP Cyclosporine RS in Diluent. Use this solution promptly after preparation.

Sample solution: Nominally 1 mg/mL of cyclosporine from Oral Solution in Diluent. Use this solution promptly after preparation.

Chromatographic system

(See Chromatography (621), System Suitability.)

Mode: LC

Detector: UV 210 nm

Column: 4.6-mm × 25-cm; packing L16

Column temperature: 50°C

Flow rate: 1 mL/min

Injection volume: 10 µL

System suitability

Sample: Standard solution

Suitability requirements

Capacity factor: 3–10

Column efficiency: NLT 700 theoretical plates

Tailing factor: NMT 1.5

Relative standard deviation: NMT 1.5%

Analysis

Samples: Standard solution and Sample solution

Calculate the percentage of the labeled amount of alcohol (C₆H₁₂O₆) in the portion of Oral Solution taken:

\[
\text{Result} = \left( \frac{R_u}{R_s} \right) \times \left( \frac{C_s}{C_u} \right) \times 100
\]

\( R_u \) = peak area ratio of alcohol to \( n \)-propyl alcohol from the Sample solution

\( R_s \) = peak area ratio of alcohol to \( n \)-propyl alcohol from the Standard solution

\( C_s \) = concentration of the Standard solution (mg/mL)

\( C_u \) = concentration of the Sample solution (mg/mL)

Acceptance criteria: 80.0%–120.0% of the labeled amount

PERFORMANCE TESTS

Uniformity of Dosage Units (905): Meets the requirements for oral solution packaged in single-unit containers

Deliverable Volume (698): Meets the requirements for oral solution packaged in multiple-unit containers

Additional Requirements

Packaging and Storage: Preserve in tight containers.

USP Reference Standards (11)

USP Cyclosporine RS

Gemcitabine Hydrochloride

\chem{C_{11}H_{10}F_2N_3O_7 \cdot HCl}

C₂₉H₁₁N₁₁O₁₂

299.66

Cytidine, 2′-deoxy-2′,2′-difluoro-, monohydrochloride
2′-Deoxy-2′,2′-difluorocytidine monohydrochloride (β-isomer) [122111-03-9].

**DEFINITION**
Gemcitabine Hydrochloride contains NLT 97.5% and NMT 101.5% of gemcitabine hydrochloride (C$_{9}$H$_{11}$F$_{2}$N$_{4}$O$_{4}$·HCl), calculated on the as-is basis.

[**CAUTION**—Gemcitabine Hydrochloride is a potent cytotoxic agent. Great care should be taken to prevent inhaling particles and exposing the skin to it.]

**IDENTIFICATION**

Change to read:

- **A. SPECTROSCOPIC IDENTIFICATION TESTS** (197), *Infrared Spectroscopy*: 197A or 197K (CN 1-May-2020). Meets the requirements

- **B. IDENTIFICATION TESTS—GENERAL** (191), *Chemical Identification Tests, Chloride*: Meets the requirements

- **C. Identification Tests, Chloride**: Meets the requirements

**ASSAY**

**PROCEDURE**
Mobile phase: 13.8 g of monobasic sodium phosphate and 2.5 mL of phosphoric acid in 1 L of water. [NOTE—The pH of this solution is 2.4–2.6.]

System suitability solution: Transfer 10 mg of Gemcitabine Hydrochloride to a small vial, add 4 mL of 168 mg/mL of potassium hydroxide in methanol, cap tightly, and sonicate. Heat at 55° for 6–16 h, allow to cool, and transfer the contents to a 100-mL volumetric flask with successive washes of 1% phosphoric acid. Dilute with 1% (v/v) phosphoric acid to volume. [NOTE—This solution contains about 0.02 mg/mL of gemcitabine α-anomer.]

Standard solution: 0.1 mg/mL of Gemcitabine Hydrochloride in water

Sample solution: 0.1 mg/mL of Gemcitabine Hydrochloride in water

Chromatographic system
(See Chromatography (621), System Suitability.)

Mode: LC

Detector: UV 275 nm

Column: 4.6-mm × 25-cm; 5-µm packing L7

Flow rate: 1.2 mL/min

Injection volume: 20 µL

System suitability

Samples: System suitability solution and Standard solution

[NOTE—The relative retention times for gemcitabine α-anomer and gemcitabine are about 0.5 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 8.0 between gemcitabine α-anomer and gemcitabine, System suitability solution

Tailing factor: NMT 1.5 for the gemcitabine peak, System suitability solution

Relative standard deviation: NMT 1.0%, Standard solution

Analysis

Samples: Standard solution and Sample solution

Calculate the percentage of cytosine in the portion of Gemcitabine Hydrochloride taken:

Result = ($r_0$/$r_1$) × ($C_1$/$C_0$) × 100

$r_0$ = peak response of cyanosine from the Sample solution

$r_1$ = peak response from the Standard solution

$r_0$ = peak response of USP Gemcitabine Hydrochloride RS in the Standard solution (mg/mL)

$r_1$ = peak response of USP Gemcitabine Hydrochloride in the Sample solution (mg/mL)

Acceptance criteria: 97.5%–101.5% on the as-is basis

**IMPURITIES**

- **RESIDUE ON IGNITION** (281): NMT 0.1%

- **ORGANIC IMPURITIES**

  System suitability solution and Chromatographic system: Proceed as directed in the Assay.

  Solution A: Use the Mobile phase, prepared as directed in the Assay.

  Solution B: Methanol

  Mobile phase: See Table 1.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Solution A (%)</th>
<th>Solution B (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>97</td>
<td>3</td>
</tr>
<tr>
<td>8</td>
<td>97</td>
<td>3</td>
</tr>
<tr>
<td>13</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>20</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>25</td>
<td>97</td>
<td>3</td>
</tr>
</tbody>
</table>

Standard solution: 0.002 mg/mL each of USP Gemcitabine Hydrochloride RS and USP Cytosine RS in water

Sample solution: 2 mg/mL of Gemcitabine Hydrochloride in water

System suitability

Samples: System suitability solution and Standard solution

Suitability requirements

Resolution: NLT 8.0 between gemcitabine α-anomer and gemcitabine, System suitability solution

Tailing factor: NMT 1.5 for the gemcitabine peak, System suitability solution

Relative standard deviation: NMT 2.0%, Standard solution

Analysis

Samples: Standard solution and Sample solution

Calculate the percentage of each impurity other than cytosine in the portion of Gemcitabine Hydrochloride taken:

Result = ($r_0$/$r_2$) × ($C_2$/$C_0$) × 100

$r_0$ = peak response of each impurity from the Sample solution

$r_2$ = peak response from the Standard solution

$r_0$ = concentration of Gemcitabine Hydrochloride in the Sample solution (mg/mL)

$r_2$ = concentration of Gemcitabine Hydrochloride RS in the Standard solution (mg/mL)
Gemcitabine for Injection

DEFINITION
Gemcitabine for Injection contains an amount of gemcitabine hydrochloride equivalent to NLT 95% and NMT 105% of the labeled amount of gemcitabine (C₈H₁₃F₂N₄O₉).

[CAUTION—Gemcitabine Hydrochloride is a potent cytotoxic agent. Great care should be taken to prevent inhaling particles and exposing the skin to it.]

SPECIFIC TESTS

• OPTICAL ROTATION (781S), Procedures, Specific Rotation
  Sample solution: 10 mg/mL
  Acceptance criteria: +43° to +50° at 20°

• PH (791)
  Sample solution: 10 mg/mL
  Acceptance criteria: 2.0–3.0

• STERILITY TESTS (71), Test for Sterility of the Product to Be Examined, Membrane Filtration: Where the label states that Gemcitabine Hydrochloride is sterile, it meets the requirements.

• BACTERIAL ENDOTOXINS TEST (85): Where the label states that Gemcitabine Hydrochloride is sterile or must be subjected to further processing during the preparation of injectable dosage forms, it contains NMT 0.05 USP Endotoxin Units/mg of gemcitabine.

ADDITIONAL REQUIREMENTS

• PACKAGING AND STORAGE: Preserve in tight containers.

• LABELING: Where it is intended for use in preparing injectable dosage forms, the label states that it is sterile or must be subjected to further processing during the preparation of injectable dosage forms.

• USP REFERENCE STANDARDS (11)
  USP Cytosine RS
  2(1H)-Pyrimidinone, 4-amino-.
  C₇H₇N₂O₂ [122-09-8]
  USP Gemcitabine Hydrochloride RS

IDENTIFICATION

Change to read:

• A. SPECTROSCOPIC IDENTIFICATION TESTS (197), Ultraviolet-Visible Spectroscopy: 197Uₐ (CN 1-May-2020)
  Medium: 0.14 M phosphate buffer with a pH of 2.5 prepared as follows. Add 13.8 g of monosodium phosphate and 2.5 mL of phosphoric acid to 1 L of water.
  Sample solution: 16 µg/mL of gemcitabine in Medium

• B. The retention time of the major peak of the Sample solution corresponds to that of the Standard solution, as obtained in the Assay.

ASSAY

• PROCEDURE
  Mobile phase: 13.8 g of monosodium phosphate and 2.5 mL of phosphoric acid in 1 L of water. [NOTE—The pH of this solution is 2.4–2.6.]

  System suitability solution: Transfer 10 mg of gemcitabine hydrochloride to a small vial, add 4 mL of 168 mg/mL of potassium hydroxide in methanol, cap tightly, and sonicate. Heat at 55° for 6–16 h, allow to cool, and transfer the contents to a 100-mL volumetric flask with successive washes of 1% phosphoric acid. Dilute with 1% (v/v) phosphoric acid to volume. [NOTE—This solution contains about 0.02 mg/mL of gemcitabine α-anomer.]

  Standard solution: 0.1 mg/mL of USP Gemcitabine Hydrochloride RS in water

  Sample solution: Equivalent to 0.1 mg/mL of gemcitabine in water from Gemcitabine for Injection prepared as follows. Reconstitute a suitable number of vials with an appropriate amount of water, based on the labeled amount of gemcitabine.

  Chromatographic system
  (See Chromatography (621), System Suitability.)
  Mode: LC
  Detector: UV 275 nm
  Column: 4.6-mm x 25-cm; 5-µm packing L7
  Flow rate: 1.2 mL/min
  Injection volume: 20 µL

  System suitability
  Samples: System suitability solution and Standard solution
  [NOTE—The relative retention times for gemcitabine α-anomer and gemcitabine are about 0.5 and 1.0, respectively.]

  Suitability requirements
  Resolution: NLT 8.0 between gemcitabine α-anomer and gemcitabine, System suitability solution
  Tailing factor: NMT 1.5 for the gemcitabine peak, System suitability solution
  Relative standard deviation: NMT 1.0%, Standard solution

  Analysis
  Samples: Standard solution and Sample solution
  Calculate the percentage of the labeled amount of gemcitabine (C₈H₁₃F₂N₄O₉) in the portion of Gemcitabine for Injection taken:

  Result \(= \left(\frac{r_1}{r_2}\right) \times \left(\frac{C_{s,1}}{C_{s,2}}\right) \times \left(\frac{M_{s,1}}{M_{s,2}}\right) \times 100\)

  \(r_1\) = peak response from the Sample solution
  \(r_2\) = peak response from the Standard solution
  \(C_{s,1}\) = concentration of USP Gemcitabine Hydrochloride RS in the Standard solution (mg/mL)
  \(C_{s,2}\) = nominal concentration of gemcitabine in the Sample solution (mg/mL)
  \(M_{s,1}\) = molecular weight of gemcitabine, 263.20

Published on March 26, 2020
\[ M_{r_2} = \text{molecular weight of gemcitabine hydrochloride, } 299.66 \]

Acceptance criteria: 95%–105%

**PERFORMANCE TESTS**

- **Uniformity of Dosage Units**, Weight Variation (905):
  - Meets the requirements

**IMPURITIES**

- **Organic Impurities**
  - System suitability solution and Chromatographic system: Proceed as directed in the Assay.
  - Solution A: Use the Mobile phase as directed in the Assay.
  - Solution B: Methanol
  - Mobile phase: See Table 1.

**System Suitability**

**Samples**: System suitability solution and Standard solution

**Suitability requirements**

- Resolution: NLT 8.0 between gemcitabine \( \alpha \)-anomer and gemcitabine, System suitability solution
- Tailing factor: NMT 1.5 for the gemcitabine peak, System suitability solution
- Relative standard deviation: NMT 2.0%, Standard solution

**Analysis**

**Samples**: Standard solution and Sample solution

Calculate the percentage of cytosine, expressed as a percentage of gemcitabine hydrochloride \((\text{C}_{2}H_{4}F_{2}N_{3}O_{4} \cdot \text{HCl})\), in the portion of Gemcitabine for Injection taken:

\[
\text{Result} = (\frac{r_u}{r_s}) \times (\frac{C_u}{C_s}) \times (\frac{M_{r_1}}{M_{r_2}}) \times 100
\]

\[ r_u = \text{peak response for each impurity from the Sample solution} \]
\[ r_s = \text{peak response of gemcitabine from the Standard solution} \]
\[ C_s = \text{concentration of USP Gemcitabine Hydrochloride RS in the Standard solution (mg/mL)} \]
\[ C_u = \text{nominal concentration of gemcitabine in the Sample solution (mg/mL)} \]
\[ M_{r_1} = \text{molecular weight of gemcitabine, 263.20} \]
\[ M_{r_2} = \text{molecular weight of gemcitabine hydrochloride, 299.66} \]

Calculate the percentage of each impurity other than cytosine, expressed as a percentage of gemcitabine hydrochloride \((\text{C}_{2}H_{4}F_{2}N_{3}O_{4} \cdot \text{HCl})\), in the portion of Gemcitabine for Injection taken:

\[
\text{Result} = (\frac{r_u}{r_s}) \times (\frac{C_u}{C_s}) \times (\frac{M_{r_1}}{M_{r_2}}) \times 100
\]

**Table 1**

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Solution A (%)</th>
<th>Solution B (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>97</td>
<td>3</td>
</tr>
<tr>
<td>8</td>
<td>97</td>
<td>3</td>
</tr>
<tr>
<td>13</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>20</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>25</td>
<td>97</td>
<td>3</td>
</tr>
</tbody>
</table>

**Table 2**

<table>
<thead>
<tr>
<th>Name</th>
<th>Relative Retention Time</th>
<th>Acceptance Criteria, NMT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytosine(^a)</td>
<td>0.4</td>
<td>0.1</td>
</tr>
<tr>
<td>Gemcitabine (\alpha)-anomer(^b)</td>
<td>0.7</td>
<td>0.1</td>
</tr>
<tr>
<td>Gemcitabine</td>
<td>1.0</td>
<td>—</td>
</tr>
<tr>
<td>Any individual unspecified impurity</td>
<td>—</td>
<td>0.2</td>
</tr>
<tr>
<td>Total impurities</td>
<td>—</td>
<td>0.3</td>
</tr>
</tbody>
</table>

\(^a\) 2(1\(H\))-Pyrimidinone, 4-amino-.
\(^b\) 2'-Deoxy-2',2'-difluorocytidine (\(\alpha\)-isomer).

**SPECIFIC TESTS**

- **Particles Matter in Injections** (788): It meets the requirements for small-volume injections.
- **pH** (791)
  - Sample solution: 40 mg/mL of gemcitabine in 0.9% sodium chloride solution
  - Acceptance criteria: 2.7–3.3

**Change to read:**

- **Clarity of Solution**
  - Sample solution: Dissolve it in the solvent and at the concentration recommended in the labeling.
  - Analysis: Determine the turbidity by ratio turbidimetry within 15 min of reconstitution, corrected for a diluent blank (see \(\text{Nephelometry and Turbidimetry (855)}\)\(^\text{a}\) (CN 1-May-2019)).

Acceptance criteria: NMT 10 NTU

- **Bacterial Endotoxins Test** (85): It contains NMT 0.05 USP Endotoxin Unit/mg of gemcitabine.

**Sterility Tests** (71): It meets the requirements when tested as directed for Test for Sterility of the Product to Be Examined, Membrane Filtration.

**ADDITIONAL REQUIREMENTS**

- **Packaging and Storage**: Preserve as described in Packaging and Storage Requirements (659), Injection Packaging, Packaging for constitution. Store at controlled room temperature. Do not refrigerate after reconstitution.

- **USP Reference Standards** (11)
  - USP Cytosine RS
  - USP Gemcitabine Hydrochloride RS
Glycerin

C\textsubscript{3}H\textsubscript{8}O\textsubscript{3}  \[ \text{92.09} \]
1,2,3-Propanetriol;
Glycerol  \([\text{56-81-5}]\).

**DEFINITION**
Glycerin contains NLT 99.0% and NMT 101.0% of C\textsubscript{3}H\textsubscript{8}O\textsubscript{3},
calculated on the anhydrous basis.

**IDENTIFICATION**
[NOTE—Compliance is determined by meeting the
requirements for Identification tests A, B, and C.]

**Change to read:**

- **A. SPECTROSCOPIC IDENTIFICATION TESTS** (197), Infrared
  Spectroscopy: 197FA (CN 1-May-2020)
- **B. LIMIT OF DIETHYLENE GLYCOL AND ETHYLENE GLYCOL**
  Standard solution: 2.0 mg/mL of USP Glycerin RS,
  0.050 mg/mL of USP Ethylene Glycol RS, 0.050 mg/mL of
  USP Diethylene Glycol RS, and 0.10 mg/mL of
  2,2,2-trichloroethanol (internal standard) in methanol
  Sample solution: 50 mg/mL of Glycerin and 0.10 mg/mL
  of 2,2,2-trichloroethanol (internal standard) in methanol

**Chromatographic system**
(See Chromatography (621), System Suitability.)

**Mode:** GC

**Detector:** Flame ionization

**Column:** 0.53-mm × 30-m fused-silica analytical column
coated with 3.0-µm G43 stationary phase, and a
deactivated split liner with glass wool

**Temperature**

<table>
<thead>
<tr>
<th>Initial Temperature (°)</th>
<th>Temperature Ramp (°/min)</th>
<th>Final Temperature (°)</th>
<th>Hold Time at Final Temperature (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>—</td>
<td>100</td>
<td>4</td>
</tr>
<tr>
<td>100</td>
<td>50</td>
<td>120</td>
<td>10</td>
</tr>
<tr>
<td>120</td>
<td>50</td>
<td>220</td>
<td>6</td>
</tr>
</tbody>
</table>

**Carrier gas:** Helium

**Injection size:** 1.0 µL

**Flow rate:** 4.5 mL/min

**Injection type:** Split ratio, about 10:1

**System suitability**

**Sample:** Standard solution

[NOTE—The relative retention times for ethylene glycol,
2,2,2-trichloroethanol, diethylene glycol, and
glycerin are about 0.3, 0.6, 0.8 and 1.0, respectively.]

**Suitability requirements**

**Resolution:** NLT 1.5 between diethylene glycol and
glycerin

**Analysis**

**Sample:** Sample solution

**Acceptance criteria:** If a peak at the retention times for
the diethylene glycol or ethylene glycol is present in the Sample
solution, the peak response ratio relative to
2,2,2-trichloroethanol is NMT the peak response ratio for
diethylene glycol or ethylene glycol relative to

2,2,2-trichloroethanol in the Sample solution; NMT 0.10%
each for diethylene glycol and ethylene glycol is found.

- **C.** Examine the chromatograms obtained in Identification
test B. The retention time of the glycerin peak of the Sample solution corresponds to that obtained in the
Standard solution.

**ASSAY**

**PROCEDURE**

**Sodium periodate solution:** Dissolve 60 g of sodium
metaperiodate in sufficient water containing 120 mL of
0.1 N sulfuric acid to make 1000 mL. Do not heat to dissolve
the periodate. If the solution is not clear, pass through a
sintered-glass filter. Store the solution in a glass-stoppered,
light-resistant container. Test the suitability of this solution
as follows. Pipet 10 mL into a 250-mL volumetric flask, and
dilute with water to volume. To 550 mg of Glycerin
dissolved in 50 mL of water, add 50 mL of the diluted
periodate solution with a pipet. For a blank, pipet 50 mL of
the solution into a flask containing 50 mL of water. Allow
the solutions to stand for 30 min, then to each add 5 mL of
hydrochloric acid and 10 mL of potassium iodide TS, and
rotate to mix. Allow to stand for 5 min, add 100 mL of
water, and titrate with 0.1 N sodium thiosulfate, shaking
continuously and adding 3 mL of starch TS as the endpoint
is approached. The ratio of the volume of 0.1 N sodium
thiosulfate required for the glycerin—periodate mixture to
that required for the blank should be between 0.750 and
0.765.

**Analysis:** Transfer 400 mg of Glycerin to a 600-mL beaker,
dilute with 50 mL of water, add bromothymol blue TS, and
acidify with 0.2 N sulfuric acid to a definite green or
greenish yellow color. Neutralize with 0.05 N sodium
hydroxide to a definite blue endpoint, free from green
color. Prepare a blank containing 50 mL of water, and
neutralize in the same manner. Pipet 50 mL of the Sodium
periodate solution into each beaker, mix by swirling gently,
cover with a watch glass, and allow to stand for 30 min at
room temperature (not exceeding 35°) in the dark or in
subdued light. Add 10 mL of a mixture of equal volumes of
ethylene glycol and water, and allow to stand for 20 min.
Dilute each solution with water to 300 mL, and titrate with
0.1 N sodium hydroxide VS to a pH of 8.1 ± 0.1 for the
specimen under assay and 6.5 ± 0.1 for the blank, using a
pH meter. Each mL of 0.1 N sodium hydroxide, after
correction for the blank, is equivalent to 9.210 mg
of C\textsubscript{3}H\textsubscript{8}O\textsubscript{3}.

**Acceptance criteria:** 99.0%–101.0% on the anhydrous basis

**IMPURITIES**

**Inorganic Impurities**

- **Chloride and Sulfate, Chloride** (221): A 7.0-g portion shows
  no more chloride than corresponds to 0.10 mL of 0.20 N
  hydrochloric acid (NMT 10 ppm).
- **Chloride and Sulfate, Sulfate** (221): A 10-g portion shows
  no more sulfate than corresponds to 0.20 mL of 0.020 N
  sulfuric acid (NMT 20 ppm).
- **Residue on Ignition** (281): Heat 50 g in an open, shallow
  100-mL porcelain dish until it ignites, and allow it to burn
  without further application of heat in a place free from
drafts. Cool, moisten the residue with 0.5 mL of sulfuric
  acid, and ignite to constant weight: the weight of the
residue does not exceed 5 mg (0.01%).

**Organic Impurities**

- **Procedure 1: Related Compounds**

  **System suitability solution:** 0.5 mg/mL each of USP
  Diethylene Glycol RS and USP Glycerin RS

  **Sample solution:** 50 mg/mL of Glycerin

Published on March 26, 2020
Chromatographic system
(See Chromatography (621), System Suitability.)
Mode: GC
Detector: Flame ionization
Column: 0.53-mm × 30-m fused-silica analytical column coated with 3.0-µm G43 stationary phase, and an inlet liner having an inverted cup or spiral structure
Temperature
Injector: 220°
Detector: 250°
Column: See the temperature program table below.

<table>
<thead>
<tr>
<th>Initial Temperature (°)</th>
<th>Temperature Ramp (°/min)</th>
<th>Final Temperature (°)</th>
<th>Hold Time at Final Temperature (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>—</td>
<td>100</td>
<td>—</td>
</tr>
<tr>
<td>100</td>
<td>7.5</td>
<td>220</td>
<td>4</td>
</tr>
</tbody>
</table>

Carrier gas: Helium
Injection size: 0.5 µL
Linear velocity: 38 cm/s
Injection type: Split ratio, about 10:1
System suitability
Sample: System suitability solution
Suitability requirements
Resolution: NLT 7.0 between diethylene glycol and glycerin
Analysis
Sample: Sample solution
Calculate the percentage of each impurity, excluding any solvent peaks and diethylene glycol, in the portion of Glycerin taken:

\[
\text{Result} = \left( \frac{r_u}{r_f} \right) \times 100
\]

where

- \( r_u \) = peak response of each individual impurity from the Sample solution
- \( r_f \) = sum of the responses of all the peaks from the Sample solution

Acceptance criteria
Individual impurities: NMT 1.0%
Total impurities: NMT 0.1%

Sample: 5 g of Glycerin
Analysis: Transfer the Sample into a dry, round-bottom, 100-mL flask. Add 15 mL of morpholine, and connect the flask by a ground joint to a reflux condenser. Reflux gently for 3 h. Rinse the condenser with 10 mL of water, receiving the washings in the flask, and cautiously acidify with nitric acid. Transfer the solution to a suitable comparison tube, add 0.50 mL of silver nitrate TS, and dilute with water to 50.0 mL.
Acceptance criteria: The turbidity is not greater than that of a blank to which 0.20 mL of 0.020 N hydrochloric acid has been added, the refluxing being omitted (NMT 30 ppm of Cl).

• Procedure 3: Fatty Acids and Esters
Sample solution: Mix 50 g of Glycerin with 50 mL of freshly boiled water and 5 mL of 0.5 N sodium hydroxide VS. Boil the mixture for 5 min, cool, and add phenolphthalein TS.
Analysis: Titrate the excess alkali with 0.5 N hydrochloric acid VS. Perform a blank determination (see Titrimetry (541), Residual Titrations).
Acceptance criteria: NMT 1 mL of 0.5 N sodium hydroxide is consumed.

SPECIFIC TESTS
• COLOR: When viewed downward against a white surface in a 50-mL color-comparison tube, the color is not darker than the color of a standard made by diluting 0.40 mL of ferric chloride CS with water to 50 mL and similarly viewed in a color-comparison tube of approximately the same diameter and color as that containing the Glycerin.
• SPECIFIC GRAVITY (841): NMT 1.249
• WATER DETERMINATION, Method I (921): NMT 5.0%

ADDITIONAL REQUIREMENTS
• PACKAGING AND STORAGE: Preserve in tight containers.
• USP REFERENCE STANDARDS (11)
USP Diethylene Glycol RS
USP Ethylene Glycol RS
USP Glycerin RS
1,2,3-Propanetriol.
C₃H₆O₃ 92.10

Hexachlorophene

C₁₃H₁₂Cl₆O₂ 406.90
Phenol, 2,2'-methylenebis[3,4,6-trichloro-]; 2,2'-Methylenebis[3,4,6-trichlorophenol] [70-30-4].

DEFINITION
Hexachlorophene contains NLT 98.0% and NMT 100.5% of hexachlorophene (C₁₃H₁₂Cl₆O₂), calculated on the dried basis.

IDENTIFICATION

Change to read:

• A. ▲ SPECTROSCOPIC IDENTIFICATION Tests (197), Infrared Spectroscopy: 197Kₑ (CN 1-May-2020)
• B.
Sample solution: 5 mg of Hexachlorophene in 5 mL of alcohol
Analysis: To the Sample solution add 1 drop of ferric chloride TS.
Acceptance criteria: A transient purple color is produced immediately.

ASSAY
• PROCEDURE
Sample: 1.5 g of Hexachlorophene
Analysis: Dissolve the Sample in 25 mL of alcohol, and titrate with 0.1 N sodium hydroxide VS, determining the endpoint potentiometrically. Perform a blank determination, and make any necessary correction. Each mL of 0.1 N sodium hydroxide is equivalent to 40.69 mg of hexachlorophene (C₁₃H₁₂Cl₆O₂).
Acceptance criteria: 98.0%–100.5% on the dried basis

IMPURITIES
• RESIDUE ON IGNITION (281): NMT 0.1%
• LIMIT OF 2,3,7,8-TCDD (CAUTION—Because 2,3,7,8-tetrachlorodibenzo-p-dioxin is an extremely toxic substance, exercise all necessary precautions in the conduct of this procedure.)
Hexachlorophene Liquid Soap

DEFINITION
Hexachlorophene Liquid Soap is a solution of Hexachlorophene in a 10.0%–13.0% solution of a potassium soap. It contains, in each 100 g, NLT 225 mg and NMT 260 mg of hexachlorophene (C₁₃H₆Cl₆O₆). It may contain suitable water hardness controls.

[Note—The inclusion of nonionic detergents in Hexachlorophene Liquid Soap in amounts greater than 8% on a total weight basis may decrease the bacteriostatic activity of the Liquid Soap.]

IDENTIFICATION

A. Sample: 2 g Analysis: Pour the Sample into a beaker, and add, with stirring, dilute hydrochloric acid (1 in 100) until the mixture is just acid to litmus. To 10 mL of the mixture, in a beaker, add 10 mL of chloroform, and shake vigorously. Acceptance criteria: The chloroform layer becomes purple.

B. Sample: 2 mL of the mixture prepared in Identification test A in a test tube

Analysis: Add 2 mL of acetone to the Sample. Add 1 mL of titanium trichloride solution (1 in 5), and shake vigorously.

Acceptance criteria: A yellow oil separates.

ASSAY

PROCEDURE
Alkaline buffer: Dissolve 6.07 g of tris(hydroxymethyl) aminomethane in 900 mL of methanol. Add 25.0 mL of dilute hydrochloric acid (1 in 10), and dilute with water to 1 L.

Solution A: Nominally 0.25 mg/mL of hexachlorophene prepared as follows. Transfer a portion of Liquid Soap containing the equivalent of about 100 mg of hexachlorophene to a 100-mL volumetric flask, and add alcohol to volume. Transfer 25.0 mL of this solution to a 100-mL volumetric flask, add 90% methanol to volume, and filter if necessary.

Control: Add 10.0 mL of Solution A to a 50-mL volumetric flask, and fill to volume with 0.3 M acetic acid in 90% methanol containing 0.1% of hydrochloric acid.

Standard solution: Transfer 50 mg of USP Hexachlorophene RS to a 100-mL volumetric flask, dissolve in 10 mL of alcohol, and dilute with Alkaline buffer to volume. Preserve in a tight container.

Standard hexachlorophene solutions: To 50-mL volumetric flasks add, by pipet and in duplicate, 2-, 3-, 4-, 5-, 6-, and 7-mL portions of the Standard solution. To one flask of each pair of duplicates, add to volume acidified 90% methanol containing, in each 100 mL, 5 mL of acetic acid and 0.3 mL of hydrochloric acid. To the second flask of each pair, add Alkaline buffer to volume. Arrange the two series of standard hexachlorophene solutions in pairs according to their hexachlorophene content.

Sample solution: Add 10.0 mL of Solution A to a 50-mL volumetric flask, and fill with Alkaline buffer to volume. Blank: Corresponding acid solution from Standard hexachlorophene solutions

Instrumental conditions

Mode: UV
Analytical wavelength: 312 nm

Analysis

Samples: Control, Standard hexachlorophene solutions, Sample solution, and Blank

Determine the absorbance of the Standard hexachlorophene solutions, using the corresponding concentration of acid solution as the Blank. Plot the observed absorbance scale against the corresponding concentration of hexachlorophene, in mg/100 mL, on the abscissa scale.

Determine the absorbance of the Sample solution, using the Control as the blank. From the observed absorbance,
calculate the weight of hexachlorophene in the Liquid
Soap taken.

Acceptance criteria: 225–260 mg of hexachlorophene in
each 100 g

SPECIFIC TESTS
- **Microbial Enumeration Tests** (61) and Tests for
  Specified Microorganisms (62): It meets the
  requirements of the tests for absence of Staphylococcus
  aureus and Pseudomonas aeruginosa.
- **Water Determination**, Method II (921)
  Sample: 5 g
  Analysis: Place the Sample, quickly weighed to the nearest
centigram, in the distilling flask of the apparatus described
in the chapter. The Liquid Soap is most conveniently
weighed in a boat of metal foil, of a size that will just pass
through the neck of the flask. Place 250 mL of toluene and
10 g of anhydrous barium chloride in the flask, connect the
flask through a ground-glass joint to the distilling
apparatus, fill the receiving tube with toluene, and
determine the water as directed, beginning with “Heat the
flask gently”.
  Acceptance criteria: The volume of water found
  corresponds to between 86.5% and 90.0% by weight of the
  portion of Liquid Soap taken.
- **Alcohol-Insoluble Substances**
  Sample: 5 g
  Analysis: Place the Sample, quickly weighed, in 100 mL of
  hot neutralized alcohol. Dissolve and collect the residue, if
  any, on a tared filter, thoroughly wash it with hot
  neutralized alcohol, and dry at 105°C for 1 h. Retain this
  solution and the residue for use in the tests for Free Alkali
  Hydroxides and Alkali Carbonates.
  Acceptance criteria: The weight of the residue obtained
  does not exceed 3.0% of the weight of Liquid Soap taken.
- **Free Alkali Hydroxides**
  Sample solution: To the combined filtrate and washings
  obtained in the test for Alcohol-Insoluble Substances add
  0.5 mL of phenolphthalein TS.
  Analysis: If a pink color is produced, titrate the solution with
  0.1 N sulfuric acid VS until the pink color is just discharged.
  Each mL of 0.1 N sulfuric acid is equivalent to 5.61 mg of
  potassium hydroxide (KOH).
  Acceptance criteria: The volume of 0.1 N sulfuric acid
  consumed corresponds to NMT 0.05% of potassium
  hydroxide (KOH).
- **Alkali Carbonates**
  Sample solution: Wash the filter containing the
  alcohol-insoluble substances from the Alcohol-Insoluble
  Substances test with 50 mL of boiling water, cool, and add
  methyl orange TS.
  Analysis: Titrate the filtrate with 0.1 N sulfuric acid VS.
  Acceptance criteria: NMT 0.5 mL of 0.1 N sulfuric acid
  per g of Liquid Soap originally taken is required [0.35% calculated as potassium carbonate (K₂CO₃)].

ADDITIONAL REQUIRMENTS
- **Packaging and Storage**: Preserve in tight, light-resistant
  containers.
- **Labeling**: Solutions of higher concentrations of
  hexachlorophene and potassium soap, in which the ratios
  of these components are consistent with the official limits,
  may be labeled “For the preparation of Hexachlorophene
  Liquid Soap, USP”, provided that the label indicates that
  the Liquid Soap is a concentrate and that directions are
  given for dilution to the official strength.
- **USP Reference Standards** (11)
  USP Hexachlorophene RS

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### Hexachlorophene Cleansing Emulsion

**DEFINITION**
Hexachlorophene Cleansing Emulsion is Hexachlorophene in a
suitable aqueous vehicle. It contains NLT 90.0% and NMT
110.0% of the labeled amount of hexachlorophene
(C₁₃H₅Cl₆O₂). It contains no coloring agents.

**IDENTIFICATION**
- **A. Thin-Layer Chromatography**
  Diluent: Chloroform and methanol (1:1)
  Standard solution: 6 mg/mL of USP Hexachlorophene RS
  in Diluent
  Sample solution: Place a volume of Cleansing Emulsion,
equivalent to 150 mg of hexachlorophene, in a
glass-stoppered, 25-mL graduated cylinder. Dilute with
Diluent to volume, and allow to stand for 5 min.
  Chromatographic system
  (See Chromatography (621), Thin-Layer Chromatography.)
  Adsorbent: 0.25-mm layer of silica gel
  Application volume: 10 μL
  Developing solvent system: Toluene and glacial acetic
  acid (9:1)
  Spray reagent: Dilute nitric acid (1 in 5)

  Analysis: Develop in the Developing solvent system until
  the solvent front has moved 10 cm above the point of
  application. Remove the plate, mark the solvent front, and
evaporate the solvent in a current of warm air. Spray the
  plate with Spray reagent, and warm on a hot plate until
  yellow spots appear.
  Acceptance criteria: The Rₘ value of the principal spot of the
  Sample solution corresponds to that of the Standard
  solution.

**ASSAY**
- **PROCEDURE**
  Standard stock solution: 1 mg/mL of USP
  Hexachlorophene RS in methanol
  Standard solution: 0.03 mg/mL of USP
  Hexachlorophene RS prepared as follows. Pipet 3 mL of
  Standard stock solution into a 100-mL volumetric flask. Add
  1 mL of dilute hydrochloric acid (1 in 10), and add
  methanol to volume.
  Sample stock solution: Nominally 0.3 mg/mL of
  hexachlorophene in methanol. Filter the solution through
  paper, taking adequate precautions to prevent
evaporation.
  Sample solution: 0.03 mg/mL of hexachlorophene
  prepared as follows. Pipet a 10-mL aliquot of the Sample
  stock solution into a 100-mL volumetric flask. Add 1 mL of
dilute hydrochloric acid (1 in 10), and add methanol to
  volume.

**Instrumental conditions**
- **Mode**: UV
- **Analytical wavelength**: UV 299 nm
- **Cell**: 1 cm
- **Blank**: Methanol and hydrochloric acid (99:1)

Analysis: Concomitantly determine the absorbances of the
Sample solution and the Standard solution.
Calculate the percentage of the labeled amount of
hexachlorophene (C₁₃H₅Cl₆O₂) in the portion of Cleansing
Emulsion taken:

\[
\text{Result} = \left( \frac{A_v}{A_s} \right) \times \left( \frac{C_s}{C_v} \right) \times 100
\]

\(A_v\) = absorbance of the Sample solution
\(A_s\) = absorbance of the Standard solution
\(C_v\) = concentration of the Standard solution (mg/mL)
Hydroxychloroquine Tablets

DEFINITION
Hydroxychloroquine Sulfate Tablets contain NLT 93.0% and NMT 107.0% of the labeled amount of hydroxychloroquine sulfate (C_{18}H_{26}ClN_{3}O·H_{2}SO_{4}).

IDENTIFICATION

A. IDENTIFICATION—ORGANIC NITROGENOUS BASES (181)
Sample solution: Nominally 20 mg/mL of hydroxychloroquine sulfate in water prepared as follows. Triturate a quantity of finely powdered Tablets, equivalent to about 1 g of hydroxychloroquine sulfate, with 50 mL of water, and filter (retain the remainder of the filtrate for Identification test B).

Acceptance criteria: The clear filtrate meets the requirements.

B. IDENTIFICATION TESTS—GENERAL, Sulfate (191): The clear filtrate obtained from Identification test A meets the requirements.
• C. The retention time of the major peak of the Sample solution corresponds to that of the Standard solution, as obtained in the Assay.

ASSAY

• PROCEDURE

Mobile phase: Methanol, acetonitrile, water, and phosphoric acid (100:100:800:2). Add 96 mg of sodium 1-pentanesulfonate in the resulting solution, and filter.

Diluent: Methanol and water (1:1)

Standard stock solution: 1 mg/mL of USP Hydroxychloroquine Sulfate RS in Diluent

Standard solution: 0.05 mg/mL of USP Hydroxychloroquine Sulfate RS from the Standard stock solution in Mobile phase

System suitability stock solution: 1 mg/mL of chloroquine phosphate in methanol

System suitability solution: Transfer 5.0 mL of the System suitability stock solution to a 100-mL volumetric flask, add 5.0 mL of Standard stock solution, and dilute with Mobile phase to volume.

Sample stock solution: Nominally 1 mg/mL of hydroxychloroquine sulfate prepared as follows. Transfer nominally 200 mg of hydroxychloroquine sulfate, from finely powdered Tablets (NLT 20), to a 200-mL volumetric flask. Add 150 mL of Diluent, and mix. Sonicate, with intermittent shaking, for 15 min, and cool to room temperature. Dilute with Diluent to volume, and filter.

Sample solution: 0.05 mg/mL from the Sample stock solution in Mobile phase

Chromatographic system

(See Chromatography (621), System Suitability.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm × 25-cm; 5- to 10-µm packing L1

Flow rate: 1 mL/min

Injection volume: 20 µL

System suitability

Samples: Standard solution and System suitability solution

Suitability requirements

Resolution: NLT 1.8 between chloroquine and hydroxychloroquine, System suitability solution

Relative standard deviation: NMT 1.5% for replicate injections of the Standard solution

Analysis

Samples: Standard solution and Sample solution

Calculate the percentage of the labeled amount of hydroxychloroquine sulfate (C₁₆H₁₈ClN₂O₄·H₂SO₄) in the Tablets taken:

Result = \( \left( \frac{r_u}{r_s} \right) \times \left( \frac{C_s}{C_i} \right) \times 100 \)

where:

\( r_u = \) peak response from the Sample solution

\( r_s = \) peak response from the Standard solution

\( C_s = \) concentration of USP Hydroxychloroquine Sulfate RS in the Standard solution (mg/mL)

\( C_i = \) nominal concentration of hydroxychloroquine sulfate in the Samplesolution (mg/mL)

Acceptance criteria: 93.0%–107.0%

PERFORMANCE TESTS

• DISSOLUTION (711)

Medium: Water; 900 mL

Apparatus 2: 50 rpm

Time: 60 min

Standard solution: USP Hydroxychloroquine Sulfate RS in Medium

Sample solution: Pass a portion of the solution through a suitable filter. Dilute with Medium, if necessary.

Instrumental conditions

Mode: UV

Analytical wavelength: 343 nm

Analysis: Determine the percentage of the labeled amount of hydroxychloroquine sulfate (C₁₆H₁₈ClN₂O₄·H₂SO₄).

Tolerances: NLT 70% (Q) of the labeled amount of hydroxychloroquine sulfate (C₁₆H₁₈ClN₂O₄·H₂SO₄) is dissolved.

• UNIFORMITY OF DOSAGE UNITS (905): Meet the requirements

ADDITIONAL REQUIREMENTS

• PACKAGING AND STORAGE: Preserve in tight, light-resistant containers.

• USP REFERENCE STANDARDS (11)

USP Hydroxychloroquine Sulfate RS

Indomethacin

![Chemical structure of Indomethacin](image)

C₁₉H₁₈CINO₄ 357.79
1H-Indole-3-acetic acid, 1-(4-chlorobenzoyl)-5-methoxy-2-methyl-;
1-(p-Chlorobenzoyl)-5-methoxy-2-methylindole-3-acetic acid [53-86-1].

DEFINITION

Indomethacin contains NLT 98.0% and NMT 102.0% of indomethacin (C₁₉H₁₈CINO₄), calculated on the dried basis.

IDENTIFICATION

Change to read:

• A. SPECTROSCOPIC IDENTIFICATION Tests (197), Infrared Spectroscopy: 197A or 197M (CN 1-May-2020)

• B. The retention time of the indomethacin peak of the Sample solution corresponds to that of the Standard solution, as obtained in the Assay.

ASSAY

• PROCEDURE

Solution A: 0.1% formic acid

Solution B: Acetonitrile

Mobile phase: Solution A and Solution B (55:45)

Diluent: Solution A and Solution B (55:45), adjusted with 0.2 M sodium hydroxide to a pH of 8.0

System suitability solution: 2.0 mg/mL of USP Indomethacin RS, 0.002 mg/mL of USP Indomethacin Related Compound A RS, and 0.001 mg/mL of USP Indomethacin Related Compound B RS, prepared as follows. Transfer a quantity of USP Indomethacin RS to a suitable volumetric flask. Add 50% of the final volume of acetonitrile and sonicate to dissolve. Add suitable amounts of USP Indomethacin Related Compound A RS and USP Indomethacin Related Compound B RS to the resulting solution, sonicate to dissolve if necessary, and dilute with Diluent to volume.

Standard solution: 0.5 mg/mL of USP Indomethacin RS in Diluent. Sonicate to dissolve.
Sample solution: 0.5 mg/mL of Indomethacin in Diluent. Sonicate to dissolve.

Chromatographic system
(See Chromatography (621), System Suitability.)
Mode: LC
Detector: UV 240 nm
Column: 4.6-mm × 25-cm; 5-µm packing L1
Temperatures
Autosampler: 4°
Column: 30°
Flow rate: 1.5 mL/min
Injection volume: 10 µL
System suitability
Samples: System suitability solution and Standard solution
[NOTE—The relative retention times for indomethacin related compound A and indomethacin related compound B are 0.24 and 0.37, respectively.]
Suitability requirements
Resolution: NLT 2.0 between indomethacin related compound A and indomethacin related compound B
Tailing factor: NMT 2.0 for indomethacin, Standard solution
Relative standard deviation: NMT 0.73%, Standard solution
Analysis
Samples: Standard solution and Sample solution
Calculate the percentage of indomethacin (C₁₅H₁₆ClNO₄) in the portion of Indomethacin taken:
\[
\text{Result} = \left( \frac{r_U}{r_S} \right) \times \left( \frac{C_S}{C_U} \right) \times 100
\]
\[r_U = \text{peak response of indomethacin from the Sample solution}\]
\[r_S = \text{peak response of indomethacin from the Standard solution}\]
\[C_S = \text{concentration of USP Indomethacin RS in the Standard solution (mg/mL)}\]
\[C_U = \text{concentration of Indomethacin in the Sample solution (mg/mL)}\]
Acceptance criteria: 98.0%–102.0% on the dried basis

IMPURITIES
• Residue on Ignition (281): NMT 0.2%
• Organic Impurities
Solution A, Solution B, Mobile phase, Diluent, and Chromatographic system: Proceed as directed in the Assay.

Standard solution: 0.002 mg/mL of USP Indomethacin RS, 0.002 mg/mL of USP Indomethacin Related Compound A RS, and 0.01 mg/mL of USP Indomethacin Related Compound B RS in Diluent. Sonicate to dissolve.

Sample solution: 2.0 mg/mL of Indomethacin, prepared as follows. Transfer a quantity of Indomethacin to a suitable volumetric flask. Add 50% of the final volume of acetonitrile and sonicate to dissolve. Dilute with Diluent to volume.

System suitability
Sample: Standard solution
Suitability requirements
Resolution: NLT 2.0 between indomethacin related compound A and indomethacin related compound B
Tailing factor: NMT 2.0 for indomethacin related compound A, indomethacin related compound B, and indomethacin
Relative standard deviation: NMT 5.0% for indomethacin related compound A, indomethacin related compound B, and indomethacin
Analysis
Samples: Standard solution and Sample solution
Calculate the percentage of indomethacin related compound A and indomethacin related compound B in the portion of Indomethacin taken:
\[
\text{Result} = \left( \frac{r_A}{r_{AB}} \right) \times \left( \frac{C_{AB}}{C_A} \right) \times 100
\]
\[r_A = \text{peak response of indomethacin related compound A from the Sample solution}\]
\[r_{AB} = \text{peak response of indomethacin related compound A and indomethacin related compound B from the Standard solution}\]
\[C_{AB} = \text{concentration of USP Indomethacin Related Compound A RS or USP Indomethacin Related Compound B RS in the Standard solution (mg/mL)}\]
\[C_A = \text{concentration of Indomethacin in the Sample solution (mg/mL)}\]
Acceptance criteria: See Table 1.

### Table 1

<table>
<thead>
<tr>
<th>Name</th>
<th>Relative Retention Time</th>
<th>Acceptance Criteria, NMT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indomethacin related compound A</td>
<td>0.24</td>
<td>0.15</td>
</tr>
<tr>
<td>Indomethacin related compound B</td>
<td>0.37</td>
<td>0.5</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>1.0</td>
<td>—</td>
</tr>
<tr>
<td>Any individual unspecified Impurity</td>
<td>—</td>
<td>0.10</td>
</tr>
<tr>
<td>Total Impurities</td>
<td>—</td>
<td>1.0</td>
</tr>
</tbody>
</table>

SPECIFIC TESTS
• Loss on Drying (731)
Analysis: Dry at a pressure below 5 mm of mercury at 100° for 2 h.
Acceptance criteria: NMT 0.5%

ADDITIONAL REQUIREMENTS
• Packaging and Storage: Preserve in well-closed, light-resistant containers.
• USP Reference Standards (11)
  - USP Indomethacin RS
  - USP Indomethacin Related Compound A RS
    (5-Methoxy-2-methylindol-3-yl)acetic acid. C_{15}H_{16}NO₄ 219.24
  - USP Indomethacin Related Compound B RS
    4-Chlorobenzoic acid. C_{6}H_{4}ClO₂ 156.57

Published on March 26, 2020
**Indomethacin Sodium**

C_{19}H_{18}CINaO_4 · 3H_2O  433.82
1H-Indole-3-acetic acid, 1-(4-chlorobenzoyl)-5-methoxy-2-
methyl-, sodium salt, trihydrate; Sodium 1-(p-chlorobenzoyl)-5-methoxy-2-methylindole-3-
acete, trihydrate [74252-25-8].

Anhydrous  379.78

**DEFINITION**
Indomethacin Sodium contains NLT 98.0% and NMT 102.0%
of indomethacin sodium (C_{19}H_{18}CINaO_4), calculated on the
dried basis.

**IDENTIFICATION**

• **A.**
  Sample: A small quantity
  Analysis: Ignite the Sample on a platinum wire in a
  nonluminous flame.
  **Acceptance criteria:** An intense yellow flame is produced.

• **B.**
  The retention time of the major peak of the **Sample**
  solution corresponds to that of the **Standard solution**, as
  obtained in the **Assay**.

• **C.**
  The UV-Vis spectrum of the major peak of the **Sample**
  solution corresponds to that of the **Standard solution**, as
  obtained in the **Assay**.

**ASSAY**

• **PROCEDURE**
  **Solution A:** 0.1% formic acid in water
  **Solution B:** 0.025% formic acid in acetonitrile
  **Mobile phase:** See **Table 1**.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Solution A (%)</th>
<th>Solution B (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>70</td>
<td>30</td>
</tr>
<tr>
<td>2</td>
<td>70</td>
<td>30</td>
</tr>
<tr>
<td>20</td>
<td>15</td>
<td>85</td>
</tr>
<tr>
<td>25</td>
<td>15</td>
<td>85</td>
</tr>
<tr>
<td>26</td>
<td>70</td>
<td>30</td>
</tr>
<tr>
<td>30</td>
<td>70</td>
<td>30</td>
</tr>
</tbody>
</table>

**System suitability solution:** 0.82 mg/mL of USP
Indomethacin RS, 0.002 mg/mL of USP Indomethacin
Related Compound A RS, and 0.002 mg/mL of USP
Indomethacin Related Compound B RS in **Diluent**. Sonicate
to dissolve.

**Standard solution:** 0.82 mg/mL of USP Indomethacin RS
dissolved in a minimum quantity of acetonitrile. Dilute with
**Diluent** to volume. Sonicate to dissolve.

**Sample solution:** 1.0 mg/mL of Indomethacin Sodium
trihydrate in **Diluent**. Sonicate to dissolve.

**Chromatographic system**
(See Chromatography (621), System Suitability.)
**Mode:** LC
**Detector:** 240 nm. For **Identification C**, use a diode array
detector in the range of 190–400 nm.

**Column:** 4.6-mm × 25-cm; 5-µm packing L1
**Column temperature:** 40°
**Flow rate:** 1 mL/min
**Injection volume:** 10 µL

**System suitability**
**Samples:** **System suitability solution** and **Standard solution**
[NOTE—The relative retention times for indomethacin
related compound A and indomethacin related
compound B are 0.50 and 0.67, respectively.]

**Suitability requirements**
**Resolution:** NLT 10 between indomethacin related
compound A and indomethacin related compound B,
**System suitability solution**
**Tailing factor:** NMT 2.0 for indomethacin, **Standard**
**solution**
**Relative standard deviation:** NMT 0.73%, **Standard**
**solution**

**Analysis**
**Samples:** **Standard solution** and **Sample solution**
Calculate the percentage of indomethacin sodium
(C_{19}H_{18}CINaO_4) in the portion of Indomethacin
Sodium taken:

\[
\text{Result} = \left( \frac{r_u}{r_S} \right) \times C_u \times \left( \frac{M_{r1}}{M_{r2}} \right) \times 100
\]

**Acceptance criteria:** 98.0%–102.0% on the dried basis

**IMPURITIES**

• **LIMIT OF ACETONE**
  **Standard solution:** Transfer 1.0 mL of acetone to a 100-mL
  volumetric flask, and dilute with water to volume. Transfer
  1.0 mL of this solution to a 200-mL volumetric flask, dilute
  with water to volume, insert a stopper, and cool in an
  ice bath.
  **Sample solution:** Transfer 100 mg of Indomethacin Sodium
to a 15-mL centrifuge tube, and dissolve in 1.0 mL of cool
  water. While vortexing this solution, add 1.0 mL of 0.24 N
  hydrochloric acid, centrifuge promptly, and filter the
  supernatant. Collect the filtrate in a suitable tube, cap, and
  cool in an ice bath.
  **Chromatographic system**
  (See Chromatography (621), System Suitability.)
  **Mode:** GC
  **Detector:** Flame ionization
  **Column:** 3-mm × 1.8-m; support S3
  **Column temperature:** 165°
  **Carrier gas:** Nitrogen
  **Injection volume:** 3 µL
  **System suitability**
  **Sample:** **Standard solution**
  **Suitability requirements**
  **Capacity factor, k**: 4–7 for acetone
  **Relative standard deviation:** NMT 2.0%
  **Analysis**
  **Samples:** **Standard solution** and **Sample solution**
  [NOTE—Use the solvent flush technique, with water as
  the flushing agent, and record the chromatograms
  for 6 min.]
Calculate the percentage of acetone in the portion of Indomethacin Sodium taken:

\[
\text{Result} = \frac{S_u (10/W_u)(r_u/r_s)}{S_u = \text{specific gravity of acetone, 0.79}}
\]

\[
W_u = \text{quantity of Indomethacin Sodium taken to prepare the Sample solution (mg)}
\]

\[
r_u = \text{peak area of acetone from the Sample solution}
\]

\[
r_s = \text{peak area of acetone from the Standard solution}
\]

Acceptance criteria: NMT 0.1%

- **Organic Impurities**
  - Solution A, Solution B, Mobile phase, Diluent, and Chromatographic system: Proceed as directed in the Assay.
  - **Standard solution**: 0.002 mg/mL each of USP Indomethacin RS, USP Indomethacin Related Compound A RS, and USP Indomethacin Related Compound B RS, dissolved in a minimum quantity of acetonitrile. Dilute with Diluent to volume. Sonicate to dissolve.
  - **Sample solution**: 1.0 mg/mL of Indomethacin Sodium in Diluent. Sonicate to dissolve.
  - **System suitability**
    - **Sample**: Standard solution
    - **Suitability requirements**
      - **Resolution**: NLT 10 between indomethacin related compound A and indomethacin related compound B
      - **Relative standard deviation**: NMT 2.5% for indomethacin related compound A, indomethacin related compound B, and indomethacin
  - **Analysis**
    - **Samples**: Standard solution and Sample solution
    - Calculate the percentage of indomethacin related compound A or indomethacin related compound B in the portion of Indomethacin Sodium taken:
      
      \[
      \text{Result} = \frac{(r_u/r_s) \times (C_s/C_u)}{100}
      \]
      
      \[
r_u = \text{peak response of indomethacin related compound A or indomethacin related compound B from the Sample solution}
      \]
      
      \[
r_s = \text{peak response of the corresponding related compound from the Standard solution}
      \]
      
      \[
C_s = \text{concentration of the corresponding USP Reference Standard in the Standard solution (mg/mL)}
\]

\[
C_u = \text{concentration of Indomethacin Sodium in the Sample solution (mg/mL)}
\]

- Calculate the percentage of any individual unspecified impurity in the portion of Indomethacin Sodium taken:
  
  \[
  \text{Result} = \frac{(r_u/r_s) \times (C_s/C_u)}{100}
  \]
  
  \[
r_u = \text{peak response of any individual unspecified impurity from the Sample solution}
  \]
  
  \[
r_s = \text{peak response of indomethacin from the Standard solution}
  \]
  
  \[
C_s = \text{concentration of USP Indomethacin RS in the Standard solution (mg/mL)}
\]

\[
C_u = \text{concentration of Indomethacin Sodium in the Sample solution (mg/mL)}
\]

Acceptance criteria: See Table 2.

### Table 2

<table>
<thead>
<tr>
<th>Name</th>
<th>Relative Retention Time</th>
<th>Acceptance Criteria, NMT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indomethacin related compound A</td>
<td>0.50</td>
<td>0.2(^a)</td>
</tr>
<tr>
<td>Indomethacin related compound B</td>
<td>0.67</td>
<td></td>
</tr>
<tr>
<td>Indomethacin</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Any individual unspecified impurity</td>
<td></td>
<td>0.5</td>
</tr>
<tr>
<td>Total impurities</td>
<td></td>
<td>1.0(^b)</td>
</tr>
</tbody>
</table>

\(^a\) The sum of the percentages of indomethacin related compound A and indomethacin related compound B is NMT 0.2%.

\(^b\) Excluding the percentages for indomethacin related compound A and indomethacin related compound B.

### Specific Tests

- **Loss on Drying (731)**
  - Analysis: Dry at 100° for 2 h at a pressure not exceeding 5 mm of mercury.
  - Acceptance criteria: 11.5%–13.5%

- **Other Requirements**: Where the label states that Indomethacin Sodium is sterile, it meets the requirements for Sterility Tests (71) and for the Bacterial Endotoxins Test under Indomethacin for Injection. Where the label states that Indomethacin Sodium must be subjected to further processing during the preparation of injectable dosage forms, it meets the requirements for the Bacterial Endotoxins Test under Indomethacin for Injection.

### Additional Requirements

- **Packaging and Storage**: Preserve in well-closed, light-resistant containers.

- **Labeling**: Where it is intended for use in preparing injectable dosage forms, the label states that it is sterile or must be subjected to further processing during the preparation of injectable dosage forms.

- **USP Reference Standards (11)**
  - USP Indomethacin RS
  - USP Indomethacin Related Compound A RS
  - USP Indomethacin Related Compound B RS

### Indomethacin Suppositories

**Definition**

Indomethacin Suppositories contain NLT 90.0% and NMT 110.0% of the labeled amount of indomethacin (C<sub>17</sub>H<sub>16</sub>CINO<sub>3</sub>).

**Identification**

- **A.** The retention time of the major peak of the Sample solution corresponds to that of the Standard solution, as obtained in the Assay.
- **B.** The UV spectrum of the indomethacin peak of the Sample solution corresponds to that of the Standard solution, as obtained in the Assay.

**Assay**

- **Procedure**
  - **Solution A**: Prepare 0.1% of formic acid by diluting 1 mL of formic acid with water to 1 L.
  - **Mobile phase**: Acetonitrile and Solution A (45:55)
Diluent: Mobile phase adjusted with 0.2 M sodium hydroxide (NaOH) to a pH of 8.0
System suitability solution: 0.002 mg/mL of USP Indomethacin RS, 0.002 mg/mL of USP Indomethacin Related Compound A RS, and 0.01 mg/mL of USP Indomethacin Related Compound B RS in Diluent
Standard solution: 0.5 mg/mL of USP Indomethacin RS in Diluent. Sonicate if necessary.
Sample solution: Prepare a solution nominally equivalent to 0.5 mg/mL of Indomethacin in Diluent as follows. Mash NLT 10 Suppositories in a beaker, and heat (at about 50°) on a water bath until melted. Mix well and cool. Transfer a portion of the mass equivalent to 10 mg of indomethacin into a 20-mL volumetric flask and add 10 mL of acetonitrile. Heat in a water bath at 50° to dissolve, and dilute with Diluent to volume.
Chromatographic system
(See Chromatography (621), System Suitability.)
Mode: LC
Detector: PDA (scan 200–600). Calculations should be based on the chromatograms collected at 240 nm. For Identification test B, use spectra at the scanned range.
Column: 4.6-mm × 25-cm; 5-µm packing L1
Column temperature: 30°
Flow rate: 1.5 mL/min
Injection volume: 10 µL
System suitability
Samples: System suitability solution and Standard solution
Suitability requirements
Resolution: NLT 4 between indomethacin related compound A and indomethacin related compound B
System suitability solution
Tailing factor: NMT 1.5, Standard solution
Relative standard deviation: NMT 1.0%, Standard solution
Analysis
Samples: Standard solution and Sample solution
Calculate the percentage of the labeled amount of indomethacin (C_{19}H_{21}ClNO_{4}) in the portion of Suppositories taken:
\[ \text{Result} = \left( \frac{r_S}{r_U} \right) \times \left( \frac{C_U}{C_S} \right) \times 100 \]
where:
- \( r_U \) = peak response from the Sample solution
- \( r_S \) = peak response from the Standard solution
- \( C_S \) = concentration of USP Indomethacin RS in the Standard solution (mg/mL)
- \( C_U \) = nominal concentration of indomethacin in the Sample solution (mg/mL)

Acceptance criteria: 90.0%–110.0%

PERFORMANCE TESTS

• DISSOLUTION (711)
  Medium: 0.1 M, pH 7.2 phosphate buffer (see Reagents, Indicators, and Solutions—Buffer Solutions); 900 mL
  Apparatus 2: 50 rpm
  Time: 60 min
  Standard solution: USP Indomethacin RS at a known concentration in Medium
  Sample solution: Proceed as directed for sample per Dissolution (711). Dilute with Medium as needed.

Instrumental conditions
Mode: UV
Analytical wavelength: 320 nm
Analysis
Samples: Standard solution and Sample solution
Tolerances: NLT 75% (Q) of the labeled amount of indomethacin (C_{19}H_{21}ClNO_{4}) is dissolved.

• UNIFORMITY OF DOSAGE UNITS (905)
  Diluent: Methanol and glacial acetic acid (199:1)
  Standard solution: 25 µg/mL of USP Indomethacin RS in Diluent
  Sample solution: Place 1 Suppository into a 100-mL volumetric flask containing 80 mL of Diluent, shake by mechanical means until the Suppository is dissolved, and dilute with Diluent to volume. Filter a portion of the solution, discarding the first 15 mL of the filtrate, and dilute a volume of the clear filtrate with the Diluent to obtain a solution having a concentration of 25 µg/mL of indomethacin.
  Instrumental conditions
  Mode: UV
  Detector: 320 nm
  Blank: Diluent
  Analysis
  Samples: Standard solution and Sample solution
  Calculate the percentage of the labeled amount of indomethacin (C_{19}H_{21}ClNO_{4}) in the Suppository taken:
  \[ \text{Result} = \left( \frac{A_U}{A_S} \right) \times \left( \frac{C_S}{C_U} \right) \times 100 \]
  where:
  - \( A_U \) = absorbance of the Sample solution
  - \( A_S \) = absorbance of the Standard solution
  - \( C_S \) = concentration of USP Indomethacin RS in the Standard solution (µg/mL)
  - \( C_U \) = nominal concentration of indomethacin in the Sample solution (µg/mL)
  Acceptance criteria: Meet the requirements in Uniformity of Dosage Units (905)

IMPURITIES

Delete the following:

- ORGANIC IMPURITIES
  Mobile phase, Diluent, and Chromatographic system:
  Proceed as directed in the Assay.
  Standard solution: 0.002 mg/mL of USP Indomethacin RS, 0.002 mg/mL of USP Indomethacin Related Compound A RS, and 0.01 mg/mL of USP Indomethacin Related Compound B RS in Diluent
  Sample solution: Prepare a solution nominally equivalent to 2.0 mg/mL of indomethacin in Diluent as follows. Mash NLT 10 Suppositories in a beaker, and heat (at about 50°) on a water bath until melted. Mix well and cool. Transfer a portion of the mass equivalent to 40 mg of indomethacin into a 20-mL volumetric flask and add 10 mL of acetonitrile. Heat in a water bath at 50° to dissolve, and dilute with Diluent to volume.
  System suitability
  Sample: Standard solution
  [NOTE—See Table 1 for the relative retention times.]
  Suitability requirements
  Resolution: NLT 4 between indomethacin related compound A and indomethacin related compound B
  Relative standard deviation: NMT 2.8%
  Analysis
  Samples: Standard solution and Sample solution
  Calculate the percentage of indomethacin related compound A and indomethacin related compound B in the portion of Suppositories taken:
  \[ \text{Result} = \left( \frac{r_A}{r_U} \right) \times \left( \frac{C_A}{C_S} \right) \times 100 \]
  where:
  - \( r_A \) = peak response of the Standard solution
  - \( r_U \) = peak response of the Sample solution
  - \( C_A \) = concentration of USP Indomethacin RS in the Standard solution (µg/mL)
  - \( C_S \) = concentration of USP Indomethacin Related Compound A RS in the Sample solution (µg/mL)
  - \( C_U \) = concentration of USP Indomethacin Related Compound B RS in the Sample solution (µg/mL)
  Acceptance criteria: Meet the requirements in Organic Impurities (910)

Published on March 26, 2020
Indomethacin Capsules

**DEFINITION**
Indomethacin Capsules contain NLT 90.0% and NMT 110.0% of the labeled amount of indomethacin (C13H18CINO₄).

**REFERENCE**
• USP R

**ASSAY**

**PROCEDURE**
Solution A: Phosphoric acid and water (2:1000)
Mobile phase: Acetonitrile and Solution A (45:55)

**Diluent**: Mobile phase adjusted with 0.2 N sodium hydroxide to a pH of 6.0

**Standard stock solution**: 1 mg/mL of USP Indomethacin RS prepared as follows. Transfer a suitable quantity of USP Indomethacin RS to an adequate volumetric flask, add 50% of the final volume of acetonitrile, and dilute with Diluent to final volume.

**Standard solution**: 0.04 mg/mL of USP Indomethacin RS in Diluent from Standard stock solution

**Sample stock solution**: Nominally 1 mg/mL of indomethacin prepared as follows. Transfer a suitable portion of the contents from NLT 20 Capsules to an adequate volumetric flask, add 50% of the final volume of acetonitrile, and sonicate for about 10 min with intermittent shaking. Add about 25% of the final volume of Diluent and sonicate for about 10 min with intermittent shaking. Dilute with Diluent to final volume and mix. Allow the resulting mixture to stand until a clear supernatant is obtained. Use the clear supernatant.

**Sample solution**: Nominally 0.04 mg/mL of indomethacin in Diluent from Sample stock solution. Pass through a suitable filter of 0.45-µm pore size.

**Chromatographic system**
(See Chromatography (621), System Suitability.)
**Mode**: LC
**Detector**: UV 240 nm
**Column**: 4.6-mm × 25-cm; 5-µm packing L1
**Column temperature**: 30°
**Flow rate**: 1.5 mL/min
**Injection volume**: 25 µL
**Run time**: NLT 2 times the retention time of indomethacin

**System suitability**
**Sample**: Standard solution
**Suitability requirements**
**Tailing factor**: NMT 2.0

**Analysis**

**Samples**: Standard solution and Sample solution

Calculate the percentage of the labeled amount of indomethacin (C13H18CINO₄) in the portion of Capsules taken:

\[
\text{Result} = \left( \frac{r_i}{r_s} \right) \times \left( \frac{C_i}{C_s} \right) \times 100
\]

where:
- \( r_i \) = peak response of unspecified impurity from the Sample solution
- \( r_s \) = peak response of unspecified impurity from the Standard solution
- \( C_i \) = concentration of unspecified impurity in the Sample solution
- \( C_s \) = concentration of the corresponding USP Indomethacin Related Compound A or USP Indomethacin Related Compound B in the Sample solution
- \( C \) = nominal concentration of indomethacin in the Sample solution
- \( S \) = peak response of indomethacin related compound A or indomethacin related compound B from the Sample solution
- \( U \) = peak response of indomethacin related compound A or indomethacin related compound B from the Standard solution

**ADDITIONAL REQUIREMENTS**

- **PACKAGING AND STORAGE**: Preserve in well-closed containers at controlled room temperature.
- **USP REFERENCE STANDARDS** (11)
  - USP Indomethacin RS
  - USP Indomethacin Related Compound A RS
  - 5-Methoxy-2-methyl-3-indoleacetic acid.
  - \( C_{13}H_{18}NO_4 \) 219.24
  - USP Indomethacin Related Compound B RS
  - 4-Chlorobenzoic acid.
  - \( C_{13}H_{18}CINO_4 \) 156.57

**IDENTIFICATION**

- **A.**
  **Standard**: A solution of 25 mg of USP Indomethacin RS in 5 mL of acetone recrystallized and prepared similarly as for the Sample.
  **Sample**: Shake a portion of the contents of Capsules, nominally equivalent to about 50 mg of indomethacin, with 10 mL of acetone for about 2 min, and filter. Transfer 5 mL of the filtrate to a stoppered flask, add 20 mL of water, and shake for about 2 min until a precipitate forms and crystallizes. Filter, and collect the crystals. Dry the crystals in air, then dry at a pressure below 5 mm of mercury at 100° for 2 h.
  **Acceptance criteria**: The IR absorption spectrum of a potassium bromide dispersion of the Sample exhibits maxima only at the same wavelengths as those of the Standard.
- **B.** The retention time of the indomethacin peak of the Sample solution corresponds to that of the Standard solution, as obtained in the Assay.

**Table 1**

<table>
<thead>
<tr>
<th>Name</th>
<th>Relative Retention Time</th>
<th>Acceptance Criteria, NMT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indomethacin related compound A</td>
<td>0.15</td>
<td>0.1</td>
</tr>
<tr>
<td>Indomethacin related compound B</td>
<td>0.25</td>
<td>0.5</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Any other individual, unidentified</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total impurities</td>
<td></td>
<td>2.04 (06/01/2019)</td>
</tr>
</tbody>
</table>

**System suitability**
**Sample**: Standard solution
**Suitability requirements**
**Tailing factor**: NMT 2.0

**Analysis**

- **Samples**: Standard solution and Sample solution

Calculate the percentage of the labeled amount of indomethacin (C13H18CINO₄) in the portion of Capsules taken:

\[
\text{Result} = \left( \frac{r_i}{r_s} \right) \times \left( \frac{C_i}{C_s} \right) \times 100
\]
The UV absorption spectrum of the indomethacin peak exhibits maxima and minima at the same wavelengths as those of the Standard solution, as obtained in the Assay.

**ADDITIONAL REQUIREMENTS**

**Packaging and Storage:** Preserve in well-closed containers.

**USP Reference Standards (11)**

USP Indomethacin RS

USP Indomethacin Related Compound A RS 2-(5-Methoxy-2-methyl-1H-indol-3-yl)acetic acid. C_{13}H_{15}NO_3 219.24

USP Indomethacin Related Compound B RS 4-Chlorobenzoic acid. C_{7}H_{5}ClO_2 156.57

### Indomethacin for Injection

**Definition**

Indomethacin for Injection contains an amount of Indomethacin Sodium equivalent to NLT 90.0% and NMT 110.0% of the labeled amount of indomethacin (C_{13}H_{15}ClNO_3).

**Identification**

**A.** The retention time of the major peak of the Sample solution corresponds to that of the Standard solution, as obtained in the Assay.

**B.** The UV absorption spectrum of the indomethacin peak of the Sample solution exhibits maxima and minima at the same wavelengths as those of the Standard solution, as obtained in the Assay.

---

**Table 1**

<table>
<thead>
<tr>
<th>Name</th>
<th>Relative Retention Time</th>
<th>Relative Response Factor</th>
<th>Acceptance Criteria, NMT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indomethacin related compound A</td>
<td>0.3</td>
<td>0.8</td>
<td>0.20</td>
</tr>
<tr>
<td>Indomethacin related compound B</td>
<td>0.4</td>
<td>2.1</td>
<td>0.20</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>1.0</td>
<td>1.0</td>
<td>—</td>
</tr>
<tr>
<td>Any unspecified degradation product</td>
<td>—</td>
<td>—</td>
<td>0.20</td>
</tr>
<tr>
<td>Total degradation products</td>
<td>—</td>
<td>—</td>
<td>1.0</td>
</tr>
</tbody>
</table>
ASSAY

• PROCEDURE
  Mobile phase: Methanol, phosphoric acid, and water (600:1:400)
  Diluent: Acetonitrile, phosphoric acid, and water (300:1:700)
  Standard solution: 0.1 mg/mL of USP Indomethacin RS prepared as follows. Dissolve a suitable amount of USP Indomethacin RS with 30% of the final volume of acetonitrile, and dilute with water to volume.
  Sample stock solution: Nominally 0.5 mg/mL of indomethacin in Diluent from NLT 10 containers of Indomethacin for Injection
  Sample solution: Nominally 0.1 mg/mL of indomethacin in Diluent from the Sample stock solution. Pass through a filter of 0.5-μm or finer pore size. Use the filtrate.

Chromatographic system
(See Chromatography (621), System Suitability.)
Mode: LC
Detector: UV 240 nm. For Identification B, use a diode array detector in the range of 200–400 nm.
Column: 3.9-mm × 30-cm; packing L1
Flow rate: 2 mL/min
Injection volume: 50 μL

System suitability
Sample: Standard solution
Suitability requirements
Tailing factor: NMT 2.0
Relative standard deviation: NMT 2.0%

Analysis
Samples: Standard solution and Sample solution
Calculate the percentage of the labeled amount of indomethacin (C₁₅H₁₆ClNO₂₄) in the portion of Indomethacin for Injection taken:

\[
\text{Result} = (r_u / r_s) \times (C_s / C_u) \times 100
\]

\( r_u \) = peak area of indomethacin from the Sample solution
\( r_s \) = peak area of indomethacin from the Standard solution
\( C_s \) = concentration of USP Indomethacin RS in the Standard solution (mg/mL)
\( C_u \) = nominal concentration of indomethacin in the Sample solution (mg/mL)

Acceptance criteria: 90.0%–110.0%

PERFORMANCE TESTS
• UNIFORMITY OF DOSAGE UNITS (905): Meets the requirements

IMPURITIES
• CONTENT OF INDOMETHACIN RELATED COMPOUND B
  Mobile phase, Diluent, Sample stock solution, Sample solution, and Chromatographic system: Proceed as directed in the Assay.
  Standard stock solution: 0.22 mg/mL of USP Indomethacin Related Compound B RS in acetonitrile
  Standard solution: 0.00044 mg/mL of USP Indomethacin Related Compound B RS prepared as follows. Transfer an adequate volume of the Standard stock solution to a suitable volumetric flask, and add 30% of the final volume of acetonitrile. Dilute with water to the final volume.

System suitability
Sample: Standard solution
Suitability requirements
Relative standard deviation: NMT 5%
Analysis
Samples: Sample solution and Standard solution

Indomethacin Oral Suspension

DEFINITION
Indomethacin Oral Suspension contains NLT 90.0% and NMT 110.0% of the labeled amount of indomethacin (C₁₅H₁₆ClNO₂₄).

IDENTIFICATION
• A. The UV absorption spectrum of the indomethacin peak of the Sample solution exhibits maxima and minima at the same wavelengths as those of the Standard solution, as obtained in the Assay.
• B. The retention time of the indomethacin peak of the Sample solution corresponds to that of the Standard solution, as obtained in the Assay.

ASSAY

• PROCEDURE
  Solution A: 0.2% (v/v) phosphoric acid in water
  Solution B: Dehydrated alcohol and butyl alcohol (80:50)
Mobile phase: Solution B and Solution A (39:61). Pass through a suitable filter of 0.5-µm or finer pore size.

Standard solution: 0.8 mg/mL of USP Indomethacin RS and 0.16 mg/mL of sorbic acid prepared as follows. Transfer appropriate amounts of USP Indomethacin RS and sorbic acid to a suitable volumetric flask. Add 20% of the total volume of Solution A and 30% of the total volume of Solution B, and sonicate for 5 min. Dilute with Solution A to the total volume.

Sample solution: Nominally 0.8 mg/mL of indomethacin prepared as follows. Transfer an appropriate volume of Oral Suspension, freshly mixed and free from air bubbles, to a suitable volumetric flask. Add 30% of Solution B, and sonicate for 10 min. Dilute with Solution A to volume, and pass through a suitable filter of 0.5-µm or finer pore size.

Chromatographic system
(See Chromatography (621), System Suitability.)

Mode: LC
Detector: UV 240 nm. For Identification A, use a diode array detector in the range of 200–400 nm.
Column: 8-mm × 10-cm; packing L1 suitable volumetric flask. Add 30% of Solution B, and sonicate for 5 min. Dilute with Solution A to the total volume.

Sample solution: Transfer to the surface of the Medium in the dissolution vessel a volume of Oral Suspension, freshly mixed and free from air bubbles, nominally equivalent to 25 mg of indomethacin. Filter portions of the solution under test, and suitably dilute with Medium if necessary.

Instrumental conditions
Mode: UV
Analytical wavelength: 320 nm

Analysis

Samples: Standard solution and Sample solution
Tolerances: NLT 80% (Q) of the labeled amount of indomethacin (C₁₉H₂₃CINO₄) is dissolved.

• UNIFORMITY OF DOSAGE UNITS (905)
  For single-unit containers
  Acceptance criteria: Meets the requirements
  For multiple-unit containers
  Acceptance criteria: Meets the requirements

IMPURITIES

• CONTENT OF INDOMETHACIN RELATED COMPOUND B
  Solution A, Solution B, Mobile phase, Sample solution, and Chromatographic system: Proceed as directed in the Assay.

Standard stock solution: 0.09 mg/mL of USP Indomethacin Related Compound B RS in Solution B

Standard solution: 0.0018 mg/mL of USP Indomethacin Related Compound B RS prepared as follows. Transfer an adequate amount of Standard stock solution to a suitable volumetric flask containing 30% of the total volume of Solution B, and dilute with Solution A to the total volume.

System suitability
Sample: Standard solution
Suitability requirements
Capacity factor, k': NLT 1.0
Relative standard deviation: NMT 2.5%

Analysis

Samples: Sample solution and Standard solution
Calculate the percentage of indomethacin related compound B in the portion of Oral Suspension taken:

Result = \( \frac{r_d}{r_s} \times \left( \frac{C_s}{C_d} \right) \times 100 \)

\( r_d \) = peak response of indomethacin related compound B from the Sample solution
\( r_s \) = peak response of indomethacin related compound B from the Standard solution
\( C_s \) = concentration of USP Indomethacin Related Compound B RS in the Standard solution (mg/mL)
\( C_d \) = nominal concentration of indomethacin in the Sample solution (mg/mL)

Acceptance criteria: NMT 0.44%

SPECIFIC TESTS
• pH (791): 2.5–5.0

ADDITIONAL REQUIREMENTS
• Packaging and Storage: Store below 30° and avoid temperatures above 50°. Preserve in tight, light-resistant containers and protect from freezing.
• USP Reference Standards (11)
  USP Indomethacin RS
  USP Indomethacin Related Compound B RS
  4-Chlorobenzoic acid.
  \( C_7H_5ClO_2 \) 156.57
Indomethacin Extended-Release Capsules

DEFINITION
Indomethacin Extended-Release Capsules contain NLT 90.0% and NMT 110.0% of the labeled amount of indomethacin (C₁₉H₁₆ClNO₄).

IDENTIFICATION

Change to read:

- A. Spectroscopic Identification Tests (197), Infrared Spectroscopy (197K)
  
  Standard: Prepare a solution of 5 mg/mL of USP Indomethacin RS in acetone. Transfer 5 mL of this solution to a stoppered flask, add 20 mL of water, and shake for 2 min until a precipitate forms and crystallizes. Filter and collect the crystals. Dry the crystals in air, then dry at a pressure below 5 mm of mercury at 100° for 2 h.

  Sample: Shake a portion of Capsule contents, nominally equivalent to 50 mg of indomethacin, with 10 mL of acetone for about 2 min, and filter. Transfer 5 mL of the filtrate to a stoppered flask, add 20 mL of water, and shake for 2 min until a precipitate forms and crystallizes. Filter and collect the crystals. Dry the crystals in air, then dry at a pressure below 5 mm of mercury at 100° for 2 h.

  Acceptance criteria: The IR absorption spectrum of the Sample exhibits maxima only at the same wavelengths as that of the Standard.

- B. The retention time of the major peak of the Sample solution corresponds to that of the Standard solution, as obtained in the Assay.

Delete the following:

- C. Sample solution: Equivalent to 1 mg/mL of indomethacin in sodium hydroxide solution (0.4 mg/mL) from powdered Capsule contents

  Analysis: Shake the Sample solution for 5 min, and filter. To 1 mL of the clear filtrate add 1 mL of 1 mg/mL sodium nitrite solution, mix, and allow to stand for 5 min. Add 0.5 mL of sulfuric acid.

  Acceptance criteria: A golden yellow color develops.

ASSAY

Change to read:

- Procedure: Methanol, water, and phosphoric acid (600: 400: 0.8)

  Diluent: Phosphoric acid and water (1:99)

  Standard solution: 0.8 mg/mL of USP Indomethacin RS, prepared as follows. Transfer a suitable quantity of USP Indomethacin RS to a suitable volumetric flask, dissolve with 60% of the flask volume of acetonitrile, and dilute with Diluent to volume.

  Sample solution: Nominally 0.75 mg/mL of indomethacin prepared as follows. Weigh and finely powder the contents of NLT 20 Capsules. Transfer a portion of the powder, nominally equivalent to 75 mg of indomethacin, to a 100-mL volumetric flask, add 40 mL of Diluent, and shake for 1 h. Sonicate for 15 min, add 40 mL of acetonitrile, sonicate for 15 min, and dilute with acetonitrile to volume. Centrifuge a portion of this solution, and use the supernatant.

  Chromatographic system:
  (See Chromatography (621), System Suitability.)

  Mode: LC
  
  Detector: UV 240 nm
  
  Column: 3.9-mm x 30-cm; 10-µm packing L1
  
  Flow rate: 2 mL/min
  
  Injection volume: 20 µL

  System suitability:
  
  Sample: Standard solution
  
  Suitability requirements
  
  Tailing factor: NMT 2.0
  
  Relative standard deviation: NMT 2.0%

  Analysis:
  
  Samples: Standard solution and Sample solution

  Calculate the percentage of the labeled amount of indomethacin (C₁₉H₁₆ClNO₄) in the portion of Capsules taken:

  \[
  \text{Result} = (r_s/r_U) \times (C_U/C_S) \times 100
  \]

  \[
  r_U = \text{peak response of indomethacin from the Sample solution}
  \]

  \[
  r_s = \text{peak response of indomethacin from the Standard solution}
  \]

  \[
  C_S = \text{concentration of USP Indomethacin RS in the Standard solution (mg/mL)}
  \]

  \[
  C_U = \text{nominal concentration of indomethacin in the Sample solution (mg/mL)}
  \]

  Acceptance criteria: 90.0%–110.0%

PERFORMANCE TESTS

Change to read:

- Dissolution (711)

  Test 1

  Medium: pH 6.2 phosphate buffer (see Reagents and Reference Tables, Solutions, Buffer Solutions); 750 mL

  Apparatus 1: 75 rpm

  Times: 1, 2, 4, 6, 12, and 24 h

  Standard solution: USP Indomethacin RS at a known concentration in Medium

  Sample solution: Pass a portion of the solution under test through a suitable filter. Dilute with Medium, if necessary.

  Instrumental conditions:
  (See Ultraviolet-Visible Spectroscopy (857).)
  
  Mode: UV
  
  Analytical wavelength: 318 nm

Analysis:

Samples: Standard solution and Sample solution

Tolerances: See Table 1.
## Table 1

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Amount Dissolved</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10%–25%</td>
</tr>
<tr>
<td>2</td>
<td>20%–40%</td>
</tr>
<tr>
<td>4</td>
<td>35%–55%</td>
</tr>
<tr>
<td>6</td>
<td>45%–65%</td>
</tr>
<tr>
<td>12</td>
<td>60%–80%</td>
</tr>
<tr>
<td>24</td>
<td>NLT 80%</td>
</tr>
</tbody>
</table>

The percentages of the labeled amount of indomethacin \((\text{C}_19\text{H}_15\text{ClNO}_3)\) dissolved at the times specified conform to *Dissolution* (711), *Acceptance Table 2*.

### Test 2

If the product complies with this test, the labeling indicates that it meets *USP Dissolution Test 2*.

**Medium:** pH 6.2 phosphate buffer (see *Reagents and Reference Tables, Solutions, Buffer Solutions*); 900 mL

**Apparatus 1:** 75 rpm (USP 1-May-2020)

**Standard solution, Sample solution, and Analysis:** Proceed as directed in *Test 1*.

**Tolerances:** See *Table 2*.

## Table 2

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Amount Dissolved</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12%–32%</td>
</tr>
<tr>
<td>2</td>
<td>27%–52%</td>
</tr>
<tr>
<td>4</td>
<td>50%–80%</td>
</tr>
<tr>
<td>12</td>
<td>NLT 80%</td>
</tr>
</tbody>
</table>

The percentages of the labeled amount of indomethacin \((\text{C}_19\text{H}_15\text{ClNO}_3)\) dissolved at the times specified conform to *Dissolution* (711), *Acceptance Table 2*.

### Test 3

If the product complies with this test, the labeling indicates that it meets *USP Dissolution Test 3*.

**Medium:** pH 6.8 phosphate buffer (see *Reagents and Reference Tables, Solutions, Buffer Solutions*); 750 mL

**Apparatus 1:** 75 rpm (USP 1-May-2020)

**Standard solution, Sample solution, and Analysis:** Proceed as directed in *Test 1*.

**Tolerances:** See *Table 3*.

## Table 3

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Amount Dissolved</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15%–40%</td>
</tr>
<tr>
<td>2</td>
<td>35%–55%</td>
</tr>
<tr>
<td>4</td>
<td>55%–75%</td>
</tr>
<tr>
<td>6</td>
<td>65%–85%</td>
</tr>
<tr>
<td>12</td>
<td>NLT 75%</td>
</tr>
<tr>
<td>24</td>
<td>NLT 85%</td>
</tr>
</tbody>
</table>

The percentages of the labeled amount of indomethacin \((\text{C}_19\text{H}_15\text{ClNO}_3)\) dissolved at the times specified conform to *Dissolution* (711), *Acceptance Table 2*.

### Test 4

If the product complies with this test, the labeling indicates that it meets *USP Dissolution Test 4*.

**Medium:** pH 6.2 phosphate buffer (see *Reagents and Reference Tables, Solutions, Buffer Solutions*); 900 mL

**Apparatus 1:** 75 rpm

### Times

1, 2, 4, 12, and 24 h

### Mobile phase

Acetonitrile and 0.1% phosphoric acid (60:40)

### Standard stock solution

Indomethacin RS \(\text{USP} \text{ClNO}_3\) prepared as follows. Transfer a suitable amount of USP Indomethacin RS into a suitable volumetric flask. Add 10% of the flask volume of acetonitrile, and sonicate to promote dissolution, if necessary. Dilute with *Medium* to volume.

### Standard solution

\((L/900)\) mg/mL of USP Indomethacin RS in *Medium* from the *Standard stock solution*, where \(L\) is the label claim, in mg

### Sample solution

Pass a portion of the solution under test through a suitable filter. Dilute with *Medium*, if necessary.

### Chromatographic system

(See *Chromatography* (621), *System Suitability*)

**Mode:** LC

**Detector:** UV 235 nm

**Column:** 4.6-mm × 100-mm; 3.5-µm packing L1

**Column temperature:** 40°

**Flow rate:** 1.2 mL/min

**Injection volume:** 10 µL

**System suitability**

**Sample:** Standard solution

**Suitability requirements**

**Relative standard deviation:** NMT 3%

### Analysis

**Samples:** Standard solution and Sample solution

Calculate the concentration \((C_i)\) of indomethacin \((\text{C}_19\text{H}_15\text{ClNO}_3)\) in the sample withdrawn from the vessel at each time point \((i)\):

\[
\text{Result} = (r_{0i}/r_{si}) \times C_i
\]

\(r_0\) = peak response of indomethacin from the *Sample solution*

\(r_s\) = peak response of indomethacin from the *Standard solution*

\(C_i\) = concentration of USP Indomethacin RS in the *Standard solution* \((\text{mg/mL})\) (USP 1-May-2020)

Calculate the percentages of the labeled amount \((Q_i)\) of indomethacin \((\text{C}_19\text{H}_15\text{ClNO}_3)\) dissolved at each time point \(i\):

\[
\text{Result}_1 = C_i \times V \times (1/L) \times 100
\]

\[
\text{Result}_2 = \left(\left\{C_i \times (V - V_0)\right\} + \left[C_i \times V_3\right]\right) \times (1/L) \times 100
\]

\[
\text{Result}_3 = \left(\left\{C_i \times (V - (i-1) \times V_3)\right\} + \left[C_i \times (i-2) + C_i \times (i-3) + ... + C_i \times V_3\right]\right) \times (1/L) \times 100
\]

\(C_i\) = concentration of indomethacin in the portion of sample withdrawn at time point \(i\) (mg/mL)

\(V\) = volume of the *Medium*, 900 mL

\(L\) = label claim of indomethacin (mg/Capsule)

\(V_0\) = volume of the *Sample solution* withdrawn from the *Medium* (mL)

**Tolerances:** See *Table 4*.

## Table 4

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Time Point (i)</th>
<th>Amount Dissolved</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>10%–30%</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>20%–40%</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>35%–55%</td>
</tr>
</tbody>
</table>

Published on March 26, 2020
The percentages of the labeled amount of indomethacin \((C_{19}H_{14}CINO_{4})\) dissolved at the times specified conform to Dissolution (711), Acceptance Table 2.

**Test 5:** If the product complies with this test, the labeling indicates that it meets USP Dissolution Test 5.

**Medium:** pH 6.2 phosphate buffer (see Reagents and Reference Tables, Solutions, Buffer Solutions); 750 mL

**Apparatus 1:** 75 rpm

**Times:** 1, 2, 4, 6, 12, and 24 h

**Standard stock solution:** 0.5 mg/mL of USP Indomethacin RS in methanol. Sonicate, if needed, to dissolve.

**Standard solution:** 0.025 mg/mL of USP Indomethacin RS from the Standard stock solution diluted in Medium. Pass through a suitable filter of 0.45-µm pore size.

**Sample solution:** Pass a portion of the solution under test through a suitable filter of 0.45-µm pore size. Dilute with Medium, if necessary.

**Instrumental conditions**

(See Ultraviolet-Visible Spectroscopy (857).)

**Mode:** UV

**Analytical wavelength:** 318 nm

**System suitability**

**Sample:** Standard solution

**Suitability requirements**

Relative standard deviation: NMT 1.0%

**Analysis:** Replace the volume of medium withdrawn for analysis with an equal volume of fresh Medium after each sampling.

**Samples:** Standard solution and Sample solution

Calculate the concentration, \(C_i\), of indomethacin \((C_{19}H_{14}CINO_{4})\) in the sample withdrawn from the vessel at each time point (\(t\)):

\[
C_i = \left( \frac{A_i}{A_j} \right) \times C_j \times D
\]

\(A_j\) = absorbance of the Sample solution at time point (\(t\))

\(A_i\) = absorbance of the Standard solution

\(C_j\) = concentration of USP Indomethacin RS in the Standard solution (mg/mL)

\(D\) = dilution factor for the Sample solution

Calculate the percentage of the labeled amount of indomethacin \((C_{19}H_{14}CINO_{4})\) dissolved at each time point (\(t\)):

\[
\text{Result}_{1} = \left( C_i \times V \right) \times \left( \frac{1}{L} \right) \times 100
\]

\[
\text{Result}_{2} = \left( \frac{C_i \times V}{C_j \times V_j} \times \frac{C_j \times V_j}{C_i \times V_j} \right) \times \left( \frac{1}{L} \right) \times 100
\]

\(C_i\) = concentration of indomethacin in the portion of sample withdrawn at time point (\(t\)) (mg/mL)

\(V\) = volume of Medium, 750 mL

\(L\) = label claim (mg/Capsule)

\(V_j\) = volume of the Sample solution withdrawn at each time point (mL)

**Tolerances:** See Table 5.

---

The percentages of the labeled amount of indomethacin \((C_{19}H_{14}CINO_{4})\) dissolved at the times specified conform to Dissolution (711), Acceptance Table 2.

**Change to read:**

- **Uniformity of Dosage Units** (905): Meets the requirements (USP 1-May-2020)

**Procedure for content uniformity**

**Solution A:** Dissolve 17.42 g of dibasic potassium phosphate in 800 mL of water, adjusting with phosphoric acid to a pH of 7.5, and diluting with water to 1000 mL (pH 7.5 phosphate buffer).

**Diluent:** Methanol and Solution A (1:1)

**Standard solution:** 25 µg/mL of USP Indomethacin RS in Diluent

**Sample solution:** Nominally 25 µg/mL of indomethacin in Diluent, prepared as follows. Transfer the contents of 1 Capsule to a 200-mL volumetric flask, and add 100 mL of Diluent. Sonicate until the contents are dispersed, dilute with Diluent to volume, and centrifuge. Dilute a portion of the clear solution with Diluent to obtain the above concentration.

**Instrumental conditions**

(See Ultraviolet-Visible Spectroscopy (857).)

**Mode:** UV

**Analytical wavelength:** 318 nm

**System suitability**

**Sample:** Standard solution and Sample solution

**Suitability requirements**

Relative standard deviation: NMT 1.0%

**Analysis**

**Samples:** Standard solution and Sample solution

Calculate the percentage of the labeled amount of indomethacin \((C_{19}H_{14}CINO_{4})\) in the Capsule taken:

\[
\text{Result} = \left( \frac{A_i}{A_j} \right) \times \left( \frac{C_i}{C_j} \right) \times 100
\]

\(A_i\) = absorbance of the Sample solution

\(A_j\) = absorbance of the Standard solution

\(C_j\) = concentration of USP Indomethacin RS in the Standard solution (µg/mL)

\(C_i\) = nominal concentration of indomethacin in the Sample solution (µg/mL)

**Impurities**

**Delete the following:**

- **Limit of 4-Chlorobenzoic Acid**

**Mobile phase, Diluent, Standard solution A, Standard solution B, Sample solution, Chromatographic system, and System suitability:** Proceed as directed in the Assay. Analysis

**Samples:** Standard solution B and Sample solution

---

Published on March 26, 2020
Using the peak responses measured and recorded in the Assay, calculate the percentage of 4-chlorobenzoic acid (C₆H₄ClO₂) in the portion of Capsules taken:

\[
\text{Result} = \left( \frac{r_d}{r_i} \times \frac{C_d}{C_i} \right) \times 100
\]

- \( r_d \) = peak response from the Sample solution
- \( r_i \) = peak response from the Standard solution
- \( C_d \) = concentration of 4-chlorobenzoic acid in the Standard solution B (mg/mL)
- \( C_i \) = measured concentration of indomethacin in the Sample solution as determined from the Assay (mg/mL)

Acceptance criteria: NMT 0.44% ▲ (USP 1-May-2020)

Add the following:

▲ ORGANIC IMPURITIES

Solution A: Dilute 1 mL of phosphoric acid with water to 1000 mL.

Solution B: Acetonitrile

Mobile phase: See Table 6.

Diluent: Acetonitrile and water (60:40)

Sensitivity solution: 0.4 µg/mL of USP Indomethacin RS in Diluent. Sonicate to dissolve if needed.

Standard solution: 0.8 µg/mL of USP Indomethacin RS, 1.1 µg/mL of USP Indomethacin Related Compound A RS, and 3.3 µg/mL of USP Indomethacin Related Compound B RS in Diluent. Sonicate to dissolve if needed.

Sample solution: Nominally 750 µg/mL of indomethacin in Diluent, prepared as follows. Transfer a suitable quantity of the contents of Capsules (NLT 20), equivalent to about 75 mg of indomethacin, to a 100-mL volumetric flask. Add about 60 mL of Diluent, shake gently for 5 min, then sonicate for about 10 min with intermittent shaking. Dilute with Diluent to volume. Pass through a suitable filter of 0.45-µm pore size.

Chromatographic system
(See Chromatography (621); System Suitability.)

[Note—Rinsing with 2 mL of methanol and water (80:20) may be used before and after injection.]

Mode: LC
Detector: UV 230 nm
Column: 4.6-mm × 25-cm; 5-µm packing L1

Temperatures
- Autosampler: 6°
- Column: 40°

Flow rate: 1 mL/min
Injection volume: 10 µL

System suitability
Samples: Sensitivity solution and Standard solution

Suitability requirements

Relative standard deviation: NMT 5.0% for indomethacin, indomethacin related compound A, and indomethacin related compound B, Standard solution

Signal-to-noise ratio: NLT 10, Sensitivity solution

Analysis

Samples: Standard solution and Sample solution

Calculate the percentages of indomethacin related compound A and indomethacin related compound B in the portion of Capsules taken:

\[
\text{Result} = \left( \frac{r_d}{r_i} \times \frac{C_d}{C_i} \right) \times 100
\]

- \( r_d \) = peak response of indomethacin related compound A or indomethacin related compound B from the Sample solution
- \( r_i \) = peak response of indomethacin related compound A or indomethacin related compound B from the Standard solution
- \( C_d \) = concentration of the corresponding USP Reference Standard in the Standard solution (µg/mL)
- \( C_i \) = nominal concentration of indomethacin in the Sample solution (µg/mL)

Calculate the percentage of any unspecified degradation product in the portion of Capsules taken:

\[
\text{Result} = \left( \frac{r_d}{r_i} \times \frac{C_d}{C_i} \right) \times 100
\]

- \( r_d \) = peak response of any unspecified degradation product from the Sample solution
- \( r_i \) = peak response of indomethacin from the Standard solution
- \( C_d \) = concentration of USP Indomethacin RS in the Standard solution (µg/mL)
- \( C_i \) = nominal concentration of indomethacin in the Sample solution (µg/mL)

Acceptance criteria: See Table 7.

<table>
<thead>
<tr>
<th>Name</th>
<th>Relative Retention Time</th>
<th>Acceptance Criteria, NMT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indomethacin related compound A</td>
<td>0.38</td>
<td>0.15</td>
</tr>
<tr>
<td>Indomethacin related compound B</td>
<td>0.59</td>
<td>0.44</td>
</tr>
<tr>
<td>Indomethacin benza-mide impurityâ†'</td>
<td>0.86</td>
<td></td>
</tr>
<tr>
<td>Indomethacin</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Indomethacin dibenzy-late impurityâ†'</td>
<td>1.07</td>
<td></td>
</tr>
<tr>
<td>Indomethacin diamide impurityâ†'</td>
<td>1.34</td>
<td></td>
</tr>
<tr>
<td>Any unspecified degradation product</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Acceptance criteria: See Table 7.
Indomethacin Compounded Topical Gel

**DEFINITION**
Indomethacin Compounded Topical Gel contains NLT 0.90 g and NMT 1.10 g of Indomethacin in 100 mL of gel. Prepare Indomethacin Compounded Topical Gel as follows.

- Indomethacin 1.0 g
- Carbomer 941 2.0 g
- Purified Water 10 mL
- Alcohol (95% ethyl alcohol), a sufficient quantity to make 100 mL

Transfer the Indomethacin to a suitable beaker, and dissolve it in 55 mL of Alcohol. Transfer this solution to a glass mortar, and slowly add the Carbomer 941 so that it is thoroughly distributed. Press out any white lumps until a smooth gel is formed. Slowly add the Purified Water with mixing. Add a sufficient quantity of Alcohol to bring to final volume, and mix. Transfer the Topical Gel to a wide-mouth container or ointment jar.

**ADDITIONAL REQUIREMENTS**
- **Packaging and Storage**: Package in tight, light-resistant, wide-mouth containers or ointment jars. Store at controlled room temperature.
- **Beyond-Use Date**: NMT 30 days after the date on which it was compounded when stored at controlled room temperature.
- **Labeling**: Label it to state that it is for topical, external use only. Label it to indicate that the container should be kept tightly closed. Label it to state the Beyond-Use Date.

### Isopropyl Alcohol

**DEFINITION**
Isopropyl Alcohol contains NLT 99.0% of isopropyl alcohol (C₃H₈O).

**IDENTIFICATION**

#### Change to read:
- **Spectroscopic Identification Tests**: Infrared Spectroscopy: 197F  
- **ASSAY**

#### Procedure
- **System suitability solution**: USP 2-Propanol System Suitability RS
- **Sample solution**: Isopropyl Alcohol (neat)
- **Chromatographic system**: See Chromatography (621), System Suitability.  
- **Mode**: GC
- **Detector**: Flame ionization
- **Column**: 0.25-mm × 60-m fused silica column, coated with a 1.4-µm film of phase G43
- **Temperatures**:  
  - **Detector**: 200°C  
  - **Injection port**: 150°C
- **Column**: See Table 1.

#### Table 1

<table>
<thead>
<tr>
<th>Initial Temperature (°C)</th>
<th>Temperature Ramp (°C/min)</th>
<th>Final Temperature (°C)</th>
<th>Hold Time at Final Temperature (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>35</td>
<td>—</td>
<td>35</td>
<td>5</td>
</tr>
<tr>
<td>35</td>
<td>1</td>
<td>45</td>
<td>—</td>
</tr>
<tr>
<td>45</td>
<td>10</td>
<td>100</td>
<td>1</td>
</tr>
</tbody>
</table>

**Carrier gas**: Helium  
**Flow rate**: 2.3 mL/min  
**Injection volume**: 1 µL  
**Injection type**: Split injection; split ratio is about 50:1.  
[Note—A 4-mm straight liner is suitable.]  
**Run time**: 22 min  
**System suitability**  
**Sample**: System suitability solution  
[Note—See Table 2.]
Table 2

<table>
<thead>
<tr>
<th>Name</th>
<th>Relative Retention Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethyl ether</td>
<td>0.7</td>
</tr>
<tr>
<td>Acetone</td>
<td>0.9</td>
</tr>
<tr>
<td>Isopropyl alcohol</td>
<td>1.0</td>
</tr>
<tr>
<td>Diisopropyl ether</td>
<td>1.4</td>
</tr>
<tr>
<td>n-Propyl alcohol (1-propanol)</td>
<td>1.5</td>
</tr>
<tr>
<td>2-Butanol</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Suitability requirements

Resolution: NLT 1.5 between acetone and isopropyl alcohol
Relative standard deviation: NMT 2.0% for the isopropyl alcohol peak
Tailing factor: NMT 2.0 for the isopropyl alcohol peak
Tailing ratio: NLT 10 for any of the following peaks: ethyl ether, acetone, isopropyl alcohol, diisopropyl ether, 1-propanol, and 2-butanol

Analysis

Sample: System suitability solution
Calculate the percentage of isopropyl alcohol (C₆H₁₄O) in the portion of Isopropyl Alcohol taken:

Result = \( \frac{r_u}{r_T} \times 100 \)

\( r_u \) = peak response of isopropyl alcohol
\( r_T \) = sum of all the peak responses

Acceptance criteria: NLT 99.0%

IMPURIES

• LIMIT OF VOLATILE RESIDUE

System suitability solution, Sample solution, Chromatographic system, and System suitability: Proceed as directed in the Assay.

Analysis

Samples: System suitability solution and Sample solution
Identify each individual impurity peak in the Sample solution based on that in the System suitability solution. Calculate the percentage of each individual impurity in the portion of Isopropyl Alcohol taken:

Result = \( \frac{r_u}{r_T} \times 100 \)

\( r_u \) = peak response of each individual impurity in the Sample solution
\( r_T \) = sum of all the peaks in the Sample solution

Acceptance criteria: See Table 3.

Table 3

<table>
<thead>
<tr>
<th>Impurity</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Each individual</td>
<td>NMT 0.1</td>
</tr>
<tr>
<td>Total</td>
<td>NMT 1.0</td>
</tr>
</tbody>
</table>

• LIMIT OF NONVOLATILE RESIDUE

Sample: 50 mL
Analysis: Evaporate the Sample in a tared porcelain dish on a steam bath to dryness, and heat at 105°C for 1 h.
Acceptance criteria: NMT 2.5 mg (0.005%)

SPECIFIC TESTS

• SPECIFIC GRAVITY (841): 0.783–0.787

Table 3

<table>
<thead>
<tr>
<th>Impurity</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lopinavir</td>
<td></td>
</tr>
</tbody>
</table>

Lopinavir

C₃₇H₄₈N₄O₅ 628.80
[1S-[1R*\(\text{R}^*\),3R*,4R*]-N-[4\((2,6-\text{Dimethylphenoxy})\) acetyl] amino]-3-hydroxy-5-phenyl-1-\(\text{(phenylmethyl)pentyl}\)-tetrahydro-\(\alpha\)-(1-methylethyl)-2-oxo-1(2H)-pyrimidineacetamide;
\((\text{s,S})\)-Tetrahydro-N-{\(\text{s,s}\)}-[\(\text{2S,3S}\)]-2-hydroxy-4-phenyl-3-[2-(2,6-xyloxy)acetamido]butyl]phenethyl]-\(\alpha\)-isopropyl-2-oxo-1(2H)-pyrimidineacetamide  \[192725-17-0\].

DEFINITION

Lopinavir contains NLT 98.0% and NMT 102.0% of lopinavir (C₃₇H₄₈N₄O₅), calculated on the anhydrous basis.

IDENTIFICATION

Change to read:

• A. SPECTROSCOPIC IDENTIFICATION TESTS (197), Infrared Spectroscopy: 197A (CN 1-May-2020)

• B. The retention time of the lopinavir peak of the Sample solution corresponds to that of the Standard solution, as obtained in the Assay.

ASSAY

• PROCEDURE

Buffer: 2.7 g/L of monobasic potassium phosphate and 0.9 g/L of dibasic potassium phosphate in water. Adjust with phosphoric acid to a pH of 6.0. Pass the solution through a suitable filter of 0.45-µm pore size.
Diluent: Acetonitrile and water (1:1)
Solution A: Acetonitrile and Buffer (9:11)
Mobile phase: Solution A
Standard solution: 0.025 mg/mL of USP Lopinavir RS in Diluent
Sample solution: 0.025 mg/mL of Lopinavir in Diluent
Chromatographic system
(See Chromatography (621), System Suitability.)
Mode: LC
Detector: UV 215 nm
Column: 4.6-mm x 25-cm; 4-µm packing L1
Column temperature: 50°C
Flow rate: 1 mL/min
Injection volume: 20 µL
Run time: 100 min

System suitability
Sample: Standard solution
Suitability requirements
Column efficiency: NLT 8000 theoretical plates
Capacity factor: NLT 15
Tailing factor: 0.8–1.5
Relative standard deviation: NLT 3.0%, Standard solution

Analysis
Samples: Standard solution and Sample solution
Calculate the percentage of each lopinavir related impurity and unidentified impurity in the portion of Lopinavir taken:

\[
\text{Result} = \left( \frac{r_U}{r_S} \right) \times \left( \frac{C_S}{C_U} \right) \times 100
\]

- \( r_U \) = peak response from the Sample solution
- \( r_S \) = peak response from the Standard solution
- \( C_S \) = concentration of USP Lopinavir RS in the Standard solution (mg/mL)
- \( C_U \) = concentration of Lopinavir in the Sample solution (mg/mL)

Acceptance criteria: 98.0%–102.0% on the anhydrous basis

IMPURITIES
- Residue on ignition (281): NMT 0.2%
- Organic impurities: Procedure 1
  [Notes—For early-eluting impurities.]
Buffer, Diluent, and Solution A: Prepare as directed in the Assay.
Solution B: Acetonitrile and Buffer (3:1)
Mobile phase: See Table 1.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Solution A (%)</th>
<th>Solution B (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>60</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>61</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>81</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>82</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>100</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

System suitability solution: 0.5 mg/mL of USP Lopinavir
System Suitability Mixture RS in Diluent
Standard solution: 0.005 mg/mL of USP Lopinavir RS in Diluent
Sample solution: 0.5 mg/mL of Lopinavir in Diluent

Chromatographic system
(See Chromatography (621), System Suitability.)
Mode: LC
Detector: UV 215 nm
Column: 4.6-mm x 25-cm; 4-µm packing L1
Column temperature: 50°C
Flow rate: 1 mL/min
Injection volume: 20 µL
Run time: 100 min

[NOTE—Data collection is only for the first 60 min. The remaining gradient steps wash out the late-eluting impurities and re-equilibrate the column.]

System suitability
Samples: System suitability solution and Standard solution
[NOTE—The relative retention times are listed in Table 2.]

Suitability requirements
Resolution: NLT 1.2 between lopinavir
- N-formylphenoxyacetamide and lopinavir
- N-acetylphenoxyacetamide, System suitability solution
Capacity factor: NLT 15, Standard solution
Column efficiency: NLT 8000, Standard solution
Tailing factor: 0.8–1.5, Standard solution
Relative standard deviation: NMT 3.0%, Standard solution

Analysis
Samples: Diluent, System suitability solution, Standard solution, and Sample solution
Calculate the percentage of each lopinavir related impurity and unidentified impurity in the portion of Lopinavir taken:

\[
\text{Result} = \left( \frac{r_U}{r_S} \right) \times \left( \frac{C_S}{C_U} \right) \times (1/F) \times 100
\]

- \( r_U \) = peak response of each impurity from the Sample solution
- \( r_S \) = peak response of lopinavir from the Standard solution
- \( C_S \) = concentration of USP Lopinavir RS in the Standard solution (mg/mL)
- \( C_U \) = concentration of Lopinavir in the Sample solution (mg/mL)
- \( F \) = relative response factor (see Table 2)

Table 2

<table>
<thead>
<tr>
<th>Name</th>
<th>Relative Retention</th>
<th>Relative Response Factor</th>
<th>Acceptance Criteria, NMT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lopinavir free amine</td>
<td>0.03</td>
<td>0.61</td>
<td>0.1</td>
</tr>
<tr>
<td>Lopinavir N-formylaminocalcohol</td>
<td>0.07</td>
<td>0.80</td>
<td>0.2</td>
</tr>
<tr>
<td>Lopinavir divalinate</td>
<td>0.10</td>
<td>0.65</td>
<td>0.1</td>
</tr>
<tr>
<td>Sulfopinavir</td>
<td>0.13</td>
<td>0.76</td>
<td>0.1</td>
</tr>
<tr>
<td>Lopinavir phenoxyacetamide</td>
<td>0.25</td>
<td>0.96</td>
<td>0.1</td>
</tr>
<tr>
<td>Lopinavir N-formylphenoxyacetamide</td>
<td>0.59</td>
<td>1.3</td>
<td>0.1</td>
</tr>
<tr>
<td>Lopinavir N-acetylphenoxyacetamide</td>
<td>0.62</td>
<td>1.2</td>
<td>0.1</td>
</tr>
<tr>
<td>Lopinavir oxazine</td>
<td>0.90</td>
<td>1.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Lopinavir</td>
<td>1.00</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Isolopinavir</td>
<td>1.10</td>
<td>0.99</td>
<td>0.2</td>
</tr>
<tr>
<td>Lopinavir 2,4-phenoxy isomer</td>
<td>1.13</td>
<td>0.97</td>
<td>0.1</td>
</tr>
<tr>
<td>Lopinavir N-valine diastereomer</td>
<td>1.25</td>
<td>1.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Z-Diacylthenediamine</td>
<td>1.28</td>
<td>1.4</td>
<td>0.1</td>
</tr>
<tr>
<td>Lopinavir (2R,4R) diastereomer</td>
<td>1.32</td>
<td>1.0</td>
<td>0.1</td>
</tr>
<tr>
<td>Lopinavir (4R) epimer</td>
<td>1.38</td>
<td>0.97</td>
<td>0.1</td>
</tr>
</tbody>
</table>
Table 2 (continued)

<table>
<thead>
<tr>
<th>Name</th>
<th>Relative Retention</th>
<th>Relative Response Factor</th>
<th>Acceptance Criteria, NMT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Any other individual impurity</td>
<td>—</td>
<td>1.0</td>
<td>0.1</td>
</tr>
</tbody>
</table>

a (S)-N-[(2S,4S,5S,5′)-5-Amino-4-hydroxy-1,6-diphenylhexan-2-yl]-3-methyl-2-[2-oxotetrahydropyrimidin-1(2H)-yl]butanamide.
b (S)-N-[(2S,4S,5S,5′)-5-Amino-4-hydroxy-1,6-diphenylhexan-2-yl]-3-methyl-2-[2-oxotetrahydropyrimidin-1(2H)-yl]butanamide.
c (2S)-2-(2,6-Dimethylphenoxy)acetamide.
d (2S)-2-(2,6-Dimethylphenoxy)acetamide.
e (2S)-2-(2,6-Dimethylphenoxy)acetamide.

**PROCEDURE**

**IMPURITIES**

- **Any other individual impurity** — 1.0 0.1
- **Organic Impurities, Procedure 2**

**BUFFER, DIULANT, AND SOLUTION A:** Prepare as directed in the Assay.

**Solution B:** Acetonitrile and Buffer (3:1)

**Mobile phase:** Solution A and Solution B (3:7)

**System suitability solution:** 0.5 mg/mL of USP Lopinavir System Suitability Mixture RS in Diluent

**Standard solution:** 0.005 mg/mL of USP Lopinavir RS in Diluent

**Sample solution:** 0.5 mg/mL in Diluent

**Chromatographic system**

*Chromatography (621), System Suitability.*

**Mode:** LC

**Detector:** UV 215 nm

**Column:** 4.6-mm x 25-cm; 4-µm packing L1

**Column temperature:** 50°C

**Flow rate:** 1 mL/min

**Injection volume:** 20 µL

**Run time:** 50 min

**System suitability**

- **Sample:** Standard solution

  **Suitability requirements**

  - **Capacity factor:** NLT 1.5
  - **Column efficiency:** NLT 3000
  - **Tailing factor:** 0.8–1.5
  - **Relative standard deviation:** NMT 3.0%

**Analysis**

- **Samples:** Diluent, System suitability solution, Standard solution, and Sample solution

**Calculate the percentage of each lopinavir related impurity and unidentified impurity in the portion of Lopinavir taken:**

\[
\text{Result} = \left(\frac{r_u}{r_s}\right) \times \left(\frac{C_u}{C_s}\right) \times (1/F) \times 100
\]

- **OTU** = peak response of each impurity from the Sample solution

- **OTS** = peak response of lopinavir from the Standard solution

- **C_u** = concentration of USP Lopinavir RS in the Sample solution (mg/mL)

- **C_s** = concentration of Lopinavir in the Sample solution (mg/mL)

- **F** = relative response factor (see Table 3)

Table 3

<table>
<thead>
<tr>
<th>Name</th>
<th>Relative Retention</th>
<th>Relative Response Factor</th>
<th>Acceptance Criteria, NMT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lopinavir</td>
<td>1.00</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Lopinavir O-acyl</td>
<td>1.49</td>
<td>0.77</td>
<td>0.1</td>
</tr>
<tr>
<td>Lopinavir (2R) epimer</td>
<td>1.91</td>
<td>1.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Lopinavir diamide</td>
<td>4.39</td>
<td>1.4</td>
<td>0.1</td>
</tr>
<tr>
<td>Lopinavir N-acyl</td>
<td>6.01</td>
<td>1.3</td>
<td>0.1</td>
</tr>
<tr>
<td>Lopinavir O-phenoxy-acetyl</td>
<td>7.14</td>
<td>1.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Lopinavir amino alcohol urea*</td>
<td>8.46</td>
<td>1.3</td>
<td>0.1</td>
</tr>
<tr>
<td>Any other individual impurity</td>
<td>—</td>
<td>1.0</td>
<td>0.1</td>
</tr>
<tr>
<td><strong>Total impurities from Procedure 1 and Procedure 2</strong></td>
<td>—</td>
<td>1.0</td>
<td>0.7%</td>
</tr>
</tbody>
</table>

a (S)-N-[(2S,4S,5S,5′)-5-Amino-4-hydroxy-1,6-diphenylhexan-2-yl]-3-methyl-2-[2-oxotetrahydropyrimidin-1(2H)-yl]butanamide.
b (S)-N-[(2S,4S,5S,5′)-5-Amino-4-hydroxy-1,6-diphenylhexan-2-yl]-3-methyl-2-[2-oxotetrahydropyrimidin-1(2H)-yl]butanamide.
c (2S)-2-(2,6-Dimethylphenoxy)acetamide.
d (2S)-2-(2,6-Dimethylphenoxy)acetamide.
e (2S)-2-(2,6-Dimethylphenoxy)acetamide.

**Acceptance criteria:** See Table 2 and Table 3.

**SPECIFIC TESTS**

**WATER DETERMINATION, Method I (921):** NMT 4.4%

**ADDITIONAL REQUIREMENTS**

**PACKAGING AND STORAGE:** Preserve in tight containers. Store at room temperature.

**USP REFERENCE STANDARDS (11)**

USP Lopinavir RS

USP Lopinavir System Suitability Mixture RS

Lopinavir System Suitability Mixture contains lopinavir N-formylphenoxyacetamide, lopinavir
N-acetylphenoxyacetamide, and several other minor
components.
Lopinavir N-formylphenoxyacetamide is \( (\text{2-}(2,6-
dimethylphenoxy)-N-H(2S,3S,5S)-5\text{-formamido-3}-
hydroxy-1,6-diphenylhexan-2-yl)\text{- acetamide} \).
\( C_{29}H_{34}N_6O_4 \) 474.59
Lopinavir N-acetylphenoxyacetamide is \( (N-[(2S,3S,5S)-5-
acetamido-3\text{-hydroxy-1,6-diphenylhexan-2-yl}]\text{-2}(2,6-
dimethylphenoxy)acetamide} \).
\( C_{30}H_{36}N_8O_4 \) 488.62

**Lopinavir and Ritonavir Tablets**

**DEFINITION**
Lopinavir and Ritonavir Tablets contain NLT 90.0% and NMT
110.0% of the labeled amounts of lopinavir \((C_{23}H_{44}N_6O_4)\)
and ritonavir \((C_{27}H_{48}N_8O_5S_2)\).

**IDENTIFICATION**
- A. The retention times of the major peaks of the Sample
  solution correspond to those of the Standard solution, as
  obtained in the Assay.

**ASSAY**
- **LOPINAVIR AND RITONAVIR**
  - Buffer 1: 4.1 g/L of monobasic potassium phosphate in
    water
  - Solution A: Acetonitrile and Buffer 1 (50:50)
  - Buffer 2: 2.1 g/L of monobasic potassium phosphate in
    water
  - Solution B: Acetonitrile and 1-butanol (13:3)
  - Solution C: Acetonitrile, 1-butanol, Buffer 1, and water
    (65:15:10:10)
  - **Standard solution:** 6.25 µg/mL of USP Ritonavir RS and 25
    µg/mL of USP Lopinavir RS in Solution A
  - **Sample solution:** Place a number of Tablets equivalent to
    1000 mg of lopinavir and 250 mg of ritonavir in a 250-mL
    volumetric flask, add 25 mL of Buffer 2, and agitate to
    dissolve the Tablet coating, if necessary. Add 100 mL of
    Solution B, and shake mechanically until the Tablets are
    dissolved. Dilute with Solution C to volume. Centrifuge a
    portion of this solution, and then further dilute with Solution
    A to a nominal concentration of 6.25 µg/mL of ritonavir and
    25 µg/mL of lopinavir.
  - **Mobile phase:** Acetonitrile, methanol, tetrahydrofuran, and
    Buffer 1 (175:100:100:625)
  - **Chromatographic system**
    - (See Chromatography (621), System Suitability.)
    - **Mode:** LC
    - **Detector:** UV 215 nm
    - **Column:** 4.6-mm × 15-cm; 5-µm packing L7
    - **Column temperature:** 40°
    - **Flow rate:** 1.5 mL/min
    - **Injection volume:** 50 µL
  - **System suitability**
    - **Sample:** Standard solution
      [Note—The elution order is ritonavir, then lopinavir.]
    - **Suitability requirements**
      - **Capacity factor:** 15–24 for the ritonavir peak
      - **Tailing factor:** 0.8–1.2 for the ritonavir peak
      - **Theoretical plates:** More than 5000 for the
        ritonavir peak
      - **Relative standard deviation:** NMT 2.0% for the ritonavir
        and lopinavir peaks
  - **Analysis**
    - **Samples:** Standard solution and Sample solution

Calculate the percentage of the labeled amount of lopinavir
\((C_{23}H_{44}N_6O_4)\) and ritonavir \((C_{27}H_{48}N_8O_5S_2)\) in the portion
of Tablets taken:

\[
\text{Result} = \left( \frac{r_u}{r_s} \right) \times \left( \frac{C_f}{C_s} \right) \times 100
\]

- \( r_u \) = peak response of lopinavir or ritonavir from the
  Sample solution
- \( r_s \) = peak response of lopinavir or ritonavir from the
  Standard solution
- \( C_f \) = concentration of USP Lopinavir RS or USP
  Ritonavir RS in the Standard solution (µg/mL)
- \( L \) = Tablet label claim for lopinavir or ritonavir (mg)
- \( D \) = dilution factor of the Sample solution
- \( V \) = volume of Medium, 900 mL

**Acceptance criteria:** 90.0%–110.0% of the labeled
amounts of lopinavir \((C_{23}H_{44}N_6O_4)\) and ritonavir
\((C_{27}H_{48}N_8O_5S_2)\)

**PERFORMANCE TESTS**
- **Dissolution**
  - **Medium:** 60 mM polyoxyethylene 10 lauryl ether (37.56 g/L)
in water; 900 mL
  - **Apparatus 2:** 75 rpm
  - **Time:** 90 min
  - **Mobile phase:** Acetonitrile and 4.1 g/L potassium phosphate
    monobasic (55:45). Adjust with phosphoric acid to an apparent
    pH of 4.0 ± 0.05.
  - **Standard solution:** Dissolve USP Lopinavir RS in methanol
to obtain a solution containing 2.6 mg/mL. Dissolve USP
  Ritonavir RS in methanol to obtain a solution containing
  1.3 mg/mL. Combine portions of these solutions to make a
  solution containing approximately 0.104 mg/mL of
  lopinavir and 0.026 mg/mL of ritonavir in Medium.
  - **Sample solutions:** Pass a portion of the solution under test
    through a suitable filter. If necessary, dilute the solution
    with Medium to obtain a final sample solution containing
    approximately 0.104 mg/mL of lopinavir and 0.026 mg/mL of
    ritonavir.
  - **Chromatographic system**
    - (See Chromatography (621), System Suitability.)
    - **Mode:** LC
    - **Detector:** UV 215 nm
    - **Column:** 4.6-mm × 15-cm; 5-µm packing L7
    - **Flow rate:** 1.5 mL/min
    - **Injection volume:** 25 µL
  - **System suitability**
    - **Sample:** Standard solution
    - **Suitability requirements**
      - **Resolution:** NLT 2.0 between lopinavir and ritonavir
      - **Tailing factor:** 0.9–1.5 for the lopinavir and ritonavir
        peaks
      - **Relative standard deviation:** NMT 2.0% for the
        lopinavir and ritonavir peaks
    - **Analysis**
      - **Samples:** Standard solution and Sample solution

Published on March 26, 2020
Tolerances: NLT 80% (Q) of the labeled amounts of lopinavir (C_{18}H_{22}N_{5}O_{4}) and ritonavir (C_{17}H_{16}N_{5}O_{5}S_{2}) are dissolved.

- **Uniformity of Dosage Units** (905): Meet the requirements

**Impurities**

- **Organic Impurities**
  - **Buffer 1**: 4.1 g/L of monobasic potassium phosphate in water
  - **Solution A**: Buffer 1 and acetonitrile (50:50)
  - **Buffer 2**: 2.1 g/L of monobasic potassium phosphate in water
  - **Solution B**: Acetonitrile, 1-butanol, and Buffer 1 (15:5:80)
  - **Solution C**: Acetonitrile, 1-butanol, Buffer 1, and water (65:15:10:10)
  - **Solution D**: Acetonitrile and 1-butanol (13:3)
  - **Buffer solution**: 3.8 g/L of monobasic potassium phosphate and 0.25 g/L of dibasic potassium phosphate in water
  - **Mobile phase**: Acetonitrile, tetrahydrofuran, 1-butanol, and Buffer solution (18:8:5:69). Adjust with 1 M phosphoric acid or 1 M potassium hydroxide, if necessary, to a pH of 6.3 ± 0.1.
  - **Standard stock solution**: 0.025 mg/mL of USP Ritonavir RS in Solution A
  - **Standard solution**: 2.5 µg/mL of USP Ritonavir RS in Solution B from Standard stock solution
  - **Ritonavir degradant identification solution**: Transfer two 5.0 mL portions of a 1 mg/mL solution of USP Ritonavir RS in Solution A to separate 50-mL volumetric flasks. Add 1 g of citric acid to one flask, and shake until dissolved. Heat both flasks at 80° for approximately 24 h. Cool the flasks, and add 13 mL of 1 N sodium hydroxide to the flask containing the citric acid. Dilute both flasks with Solution B to volume. Combine equal volumes of both solutions. This solution contains ritonavir and the ritonavir degradation products (N-deacylvaline ritonavir, hydantoin aminoalcohol, O-acyl isomer, and oxazolidinone derivative).
  - **Ritonavir related compounds identification solution**: 1 mg/mL of USP Ritonavir Related Compounds Mixture RS dissolved in Solution C and further diluted with Solution B to 0.5 mg/mL.
  - **Sample solution**: Place a number of Tablets equivalent to 1000 mg of lopinavir and 250 mg of ritonavir into a 250-mL volumetric flask. Add 25 mL of Buffer 2, and agitate to dissolve the Tablet coating, if necessary. Add 100 mL of Solution D, and shake mechanically until the Tablets are dissolved. Dilute with Solution C to volume. Centrifuge a portion of this solution, and further dilute with Solution B to a concentration of 2 mg/mL of lopinavir and 0.5 mg/mL of ritonavir.

**Chromatographic system**
(See Chromatography (621), System Suitability.)
- **Column**: 4.6-mm × 15-cm; 3-µm packing L26
- **Column temperature**: 60°
- **Detector**: UV 240 nm
- **Injection volume**: 50 µL
- **Flow rate**: 1.0 mL/min

**System suitability**
- **Samples**: Ritonavir degradant identification solution, Ritonavir related compounds identification solution, and Standard solution
- **Resolution**: NLT 1.0 between the peaks for O-acyl isomer and oxazolidinone derivative, Ritonavir degradant identification solution. NLT 0.7 between the peaks for hydroxyritonavir and hydantoin aminoalcohol, Ritonavir related compounds identification solution
- **Capacity factor**: NLT 10.8, Standard solution

**Acceptance criteria**: See Table 1. [Note—Disregard all peaks eluting before the retention time of the N-deacylvaline ritonavir peak from the Ritonavir degradant identification solution.]

**Analysis**

Calculate the percentage of each ritonavir degradation product in the Sample solution:

\[ \text{Result} = \left( \frac{r_0}{r_s} \right) \times \left( \frac{C_s}{C_U} \right) \times (1/F) \times 100 \]

\[ r_0 = \text{peak area of individual degradation product from the Sample solution} \]
\[ r_s = \text{peak response of ritonavir from the Standard solution} \]
\[ C_s = \text{concentration of USP Ritonavir RS in the Standard solution (mg/mL)} \]
\[ C_U = \text{nominal concentration of ritonavir in the Sample solution (mg/mL)} \]
\[ F = \text{relative response factor} \]

Table 1

<table>
<thead>
<tr>
<th>Name</th>
<th>Relative Retention</th>
<th>Relative Response Factor</th>
<th>Acceptance Criteria, NMT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Deacylvaline ritonavir</td>
<td>0.11</td>
<td>0.81</td>
<td>0.2</td>
</tr>
<tr>
<td>Acetamidoalcohol</td>
<td>0.15</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>2,5-Thiazolylmethyl dicarbamate</td>
<td>0.24</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Hydroxyritonavir</td>
<td>0.36</td>
<td>0.86</td>
<td>0.3</td>
</tr>
<tr>
<td>Hydantoin aminoalcohol</td>
<td>0.39</td>
<td>0.73</td>
<td>2.6</td>
</tr>
<tr>
<td>Ritonavir hydroperoxide</td>
<td>0.44</td>
<td>0.88</td>
<td>0.2</td>
</tr>
<tr>
<td>Hydantoin-oxazolidinone derivative</td>
<td>0.50</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Ethyl analog</td>
<td>0.64</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>O-Acyl isomer</td>
<td>0.74</td>
<td>1.1</td>
<td>0.2</td>
</tr>
<tr>
<td>BOC-aminoalcohol</td>
<td>0.81</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Isobutoxycarbonyl aminalcohol</td>
<td>0.81</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Oxazolidinone derivative</td>
<td>0.87</td>
<td>0.53</td>
<td>0.3</td>
</tr>
<tr>
<td>Ureidovaline isobutylerister</td>
<td>0.94</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Ritonavir</td>
<td>1.0</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>4-Hydroxy isomer</td>
<td>1.05</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>3R-Epimer</td>
<td>1.11</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Aminooalcohol urea derivative</td>
<td>1.14</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>3R,5R-Epimer</td>
<td>1.23</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>5R-Epimer</td>
<td>1.32</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Diacyl valine urea</td>
<td>1.70</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

Any unspecified impurity — 1.0 0.2
Lopinavir and Ritonavir Oral Solution

**DEFINITION**
Lopinavir and Ritonavir Oral Solution contains NLT 90.0% and NMT 110.0% of the labeled amounts of lopinavir (C_{37}H_{48}N_{6}O_{5}) and ritonavir (C_{37}H_{48}N_{6}O_{5}).

**IDENTIFICATION**

A. The retention times of the lopinavir and ritonavir peaks of the Sample solution correspond to those of the Standard solution, as obtained in the Assay.

**ASSAY**

### Lopinavir and Ritonavir Oral Solution

#### Buffer

4.1 g/L of monobasic potassium phosphate in water

**Solution A:** Acetonitrile and Buffer (65:35)

**Solution B:** Acetonitrile and Buffer (50:50)

#### Mobile phase

Acetonitrile, methanol, tetrahydrofuran, and Buffer (175:100:100:625). Separately filter the Buffer and the premixed solvents before combining them to make the Mobile phase.

**Standard stock solution:** 0.1 mg/mL each of USP Lopinavir RS and USP Ritonavir RS in Solution B

**Solution B**

0.025 mg/mL each of USP Lopinavir RS and USP Ritonavir RS in Solution B from the Standard stock solution

#### Sample stock solution

Nominally 0.05 mg/mL of lopinavir and 0.0125 mg/mL of ritonavir in Solution B from the Sample stock solution

#### Chromatographic system

(See Chromatography (621), System Suitability.)

**Mode:** LC

**Detector:** UV 215 nm

**Column:** 4.6 mm × 15 cm; 5-µm packing L7

**Column temperature:** 40°

**Flow rate:** 1.5 mL/min

**Injection volume:** 50 µL

#### System suitability

**Sample:** Standard solution

**Suitability requirements**

Tailing factor: 0.8–1.2 for the ritonavir peak

Relative standard deviation: NMT 2.0% each for ritonavir and lopinavir

#### Analysis

**Samples:** Standard solution and Sample solution

Calculate the percentages of the labeled amounts of lopinavir (C_{37}H_{48}N_{6}O_{5}) and ritonavir (C_{37}H_{48}N_{6}O_{5}) in the portion of Oral Solution taken:

\[
\text{Result} = \left( \frac{r_u}{r_s} \right) \times (C_o/C_s) \times 100
\]

\( r_u \) = peak response of the corresponding analyte from the Sample solution

\( r_s \) = peak response of the corresponding analyte from the Standard solution

\( C_s \) = concentration of USP Lopinavir RS or USP Ritonavir RS in the Standard solution (mg/mL)

\( C_o \) = nominal concentration of lopinavir or ritonavir in the Sample solution (mg/mL)

Acceptance criteria: 90.0%–110.0% of the labeled amounts of lopinavir (C_{37}H_{48}N_{6}O_{5}) and ritonavir (C_{37}H_{48}N_{6}O_{5})

#### PERFORMANCE TESTS

**DELIBERABLE VOLUME (698)**

For multiple-unit containers

Acceptance criteria: Meets the requirements

#### IMPURITIES

**Organic impurities**

**Buffer A:** 4.1 g/L of monobasic potassium phosphate in water

**Buffer B:** 3.8 g/L of monobasic potassium phosphate and 0.25 g/L of dibasic potassium phosphate in water

**Solution A:** Acetonitrile and Buffer A (50:50)
Solution B: Acetonitrile, butyl alcohol, and Buffer A (15:9:80)
Solution C: Acetonitrile and Buffer A (65:35)
Mobile phase: Acetonitrile, tetrahydrofuran, butyl alcohol, and Buffer B (18:8:5:69). Adjust with 1 M phosphoric acid or 1 M potassium hydroxide, if necessary, to a pH of 6.3.
Standard stock solution: 0.1 mg/mL each of USP Ritonavir RS and USP Hydroxyridonavir in Solution A
Standard solution: 0.01 mg/mL each of USP Ritonavir RS and USP Hydroxyridonavir from Standard stock solution in Solution B
Peak identification solution: Transfer a weighed portion of Oral Solution to a crimp-top container. Add an amount of citric acid equivalent to 1% by weight of the Oral Solution taken and mix until dissolved. Seal the container, and heat at 50°C for approximately 4 days. Use this degradation solution and follow the procedure described below in the Sample stock solution and Sample solution sections to prepare the Peak identification solution.
Sample stock solution: Transfer 5 mL of Oral Solution with the aid of several small portions of Solution C to a 100-mL volumetric flask, and dilute with Solution C to volume.
Sample solution: Dilute 25.0 mL of Sample stock solution with Solution B to 50.0 mL. Transfer 15.0 mL of this solution to a 50-mL centrifuge tube that has been previously rinsed several times with methanol and dried. Add 20.0 mL of n-heptane, and shake vigorously until a uniform emulsion is formed. Vent the tube periodically while shaking. Centrifuge the emulsion for about 5 min. Carefully remove the top heptane layer by aspiration, leaving the clear Sample solution layer. The middle viscous, cloudy layer should be considered part of the heptane layer for removal by aspiration. Precondition a strong anion-exchange cartridge (quaternary ammonium functionality on a styrene/ divinylbenzene base) with a sorbent mass of 600 mg by rinsing the cartridge with 3 mL of methanol, then 3 mL of Solution C, and repeating these rinse steps. Dry the cartridge for about 10 min with the aid of a low vacuum. Transfer 5.0 mL of the clear Sample solution to the preconditioned cartridge. With the aid of a vacuum, slowly pass the Sample solution completely through the cartridge, collect the extract in a 5-mL volumetric flask, and then dilute with Solution C to volume.
Chromatographic system
(See Chromatography (621), System Suitability.)
Mode: LC

<table>
<thead>
<tr>
<th>Name</th>
<th>Relative Retention Value (r)</th>
<th>Relative Response Factor</th>
<th>Acceptance Criteria, NMT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ureidovaline</td>
<td>0.03</td>
<td>—</td>
<td>—b</td>
</tr>
<tr>
<td>N-Deacetylvaline ritonavir</td>
<td>0.11</td>
<td>0.81</td>
<td>0.8</td>
</tr>
<tr>
<td>Glycerol carbamate analog</td>
<td>0.14</td>
<td>0.62</td>
<td>0.2</td>
</tr>
<tr>
<td>Acetamidoalcohol</td>
<td>0.15</td>
<td>—</td>
<td>—b</td>
</tr>
<tr>
<td>Hydroxypropyl carbamate analog</td>
<td>0.24</td>
<td>0.59</td>
<td>0.5</td>
</tr>
<tr>
<td>2,5-Thiazolylmethylcarbamate</td>
<td>0.24</td>
<td>—</td>
<td>—b</td>
</tr>
<tr>
<td>Hydroxyritonavir</td>
<td>0.36</td>
<td>0.86</td>
<td>0.3</td>
</tr>
<tr>
<td>Hydantoin aminoalcohol</td>
<td>0.39</td>
<td>0.73</td>
<td>0.4</td>
</tr>
<tr>
<td>Ritonavir hydroperoxide</td>
<td>0.44</td>
<td>0.88</td>
<td>0.2</td>
</tr>
<tr>
<td>Ethyl carbamate analog</td>
<td>0.45</td>
<td>0.66</td>
<td>0.7</td>
</tr>
<tr>
<td>Hydantoin-oxazolidinone derivative</td>
<td>0.30</td>
<td>—</td>
<td>—b</td>
</tr>
</tbody>
</table>

Detector: UV 215 nm and 240 nm
Column: 4.6-mm × 15-cm; 3-µm packing L26
Column temperature: 60°C
Flow rate: 1 mL/min
Injection volume: 50 µL
Run time: 2 times the retention time of lopinavir

System suitability
Sample: Standard solution
Suitability requirements
Resolution: NLT 2.5 between the ritonavir and lopinavir peaks at 215 nm
Tailing factor: 0.8–1.2 for the ritonavir peak at 240 nm
Relative standard deviation: NMT 3.0% for the ritonavir peak at 215 nm; NMT 3.0% for the ritonavir peak at 240 nm

Analysis
Samples: Standard solution, Peak identification solution, and Sample solution

To identify the ritonavir impurities, determine the relative retention value from the 240-nm chromatogram relative to ritonavir (see Table 1). The Peak identification solution may also be used to identify ritonavir degradants.

Unspecified ritonavir impurities are assigned according to the algorithm outlined in Table 3.

Acceptance criteria: See Table 1.
<table>
<thead>
<tr>
<th>Name</th>
<th>Relative Retention Value (r)</th>
<th>Relative Response Factor</th>
<th>Acceptance Criteria, NMT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethyl analogb</td>
<td>0.64</td>
<td></td>
<td></td>
</tr>
<tr>
<td>O-Acyl isomerb</td>
<td>0.74</td>
<td>1.1</td>
<td>0.2</td>
</tr>
<tr>
<td>BOC-aminalcoholh</td>
<td>0.81</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isobutoxycarbonyl aminalcoholh</td>
<td>0.81</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxazolidine derivative</td>
<td>0.87</td>
<td>0.53</td>
<td>0.2</td>
</tr>
<tr>
<td>Ureidovaline isobutyl estera</td>
<td>0.94</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ritonavir</td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-Hydroxy isomer</td>
<td>1.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3R-Epimer</td>
<td>1.11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aminolcohol urea derivative</td>
<td>1.14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3R,5R-Epimer</td>
<td>1.23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5R-Epimer</td>
<td>1.32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diacyl valine urea</td>
<td>1.70</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Any unspecified ritonavir impurity</td>
<td>—</td>
<td>1.0</td>
<td>0.2</td>
</tr>
<tr>
<td>Total ritonavir impurities, specified and unspecified</td>
<td>—</td>
<td>—</td>
<td>3.0**</td>
</tr>
</tbody>
</table>

a [N-Methyl[(2-isopropyl-4-thiazolyl)amino]carboxy]valine-valine.

b These are process impurities which are included in this table for identification only. These impurities are controlled in the drug substance. They are not to be reported for the drug product and are not included in the total impurities.

c Thiogalol-3-ylmethyl [(2S,3S,5S)-5-[(S)-2-amino-3-methylbutanamido]-3-hydroxy-1,6-diphenylhexan-2-ylcarbamate.

d Thiogalol-3-ylmethyl [(2S,3S,5S)-5-[(2S)-2-(2,3-dihydroxypropoxy)carboxyamino]-3-methylbutanamido]-3-hydroxy-1,6-diphenylhexan-2-ylcarbamate.

e Thiogalol-3-ylmethyl [(2S,3S,5S)-5-[(2S)-2-(2-hydroxypropoxy)carboxyamino]-3-methylbutanamido]-3-hydroxy-1,6-diphenylhexan-2-ylcarbamate.

f Thiogalol-3-ylmethyl [(2S,3S,5S)-5-acetamido-3-hydroxy-1,6-diphenylhexan-2-ylcarbamate.

g Thiogalol-3-ylmethyl (2S,3S,5S)-5-[(5-isobutoxycarbonylamino)-3-hydroxy-1,6-diphenylhexan-2-ylcarbamate.

h A single peak with a relative retention value of 0.44 should be reported as the ethyl carbamate analog due to possible coelution with ritonavir hydroperoxide impurity.

i Thiaolol-3-ylmethyl (2S,3S,5S)-3-hydroxy-5-[(2-[(2-hydroxypropan-2-yl)thiazol-4-yl)methyl]-3-methylureido]acetalamido]-1,6-diphenylhexan-2-ylcarbamate.

j Thiaolol-3-ylmethyl (2S,3S,5S)-3-hydroxy-5-[(S)-4-isopropyl-2,5-dioximidazolidin-1-yl]-1,6-diphenylhexan-2-ylcarbamate.

k Thiaolol-3-ylmethyl (2S,3S,5S)-3-hydroxy-5-[(S)-2-ethoxycarbonylamino]carboxylamino]-3-methylbutanamido]-3-hydroxy-1,6-diphenylhexan-2-ylcarbamate.

l Thiaolol-3-ylmethyl (2S,3S,5S)-4-benzyl-5-[(S)-2-[(2-isopropylthiazol-4-yl)methyl]-3-methylureido]oxo]oxazolidine-3-carboxylate.

m Thiaolol-3-ylmethyl (2S,3S,5S)-5-[(S)-2-[(2-ethylthiazol-4-yl)methyl]-3-methylureido]oxo]oxazolidine-3-carboxylate.

n Thiaolol-3-ylmethyl (2S,3S,5S)-5-[(S)-2-[(2-ethylthiazol-4-yl)methyl]-3-methylureido]-3-hydroxy-1,6-diphenylhexan-2-ylcarbamate.

o (S)-[(2S,3S,5S)-5-Amino-1,6-diphenyl-2-[(thiazol-5-methylthoxy)carboxyamino]hexan-3-yl]-2-[(2-isopropylthiazol-4-yl)methyl]-3-methylureido]-3-methylbutanoate.

p (S)-[(2S,3S,5S)-5-Amino-1,6-diphenyl-2-[(thiazol-5-methylthoxy)carboxyamino]hexan-3-yl]-2-[(2-isopropylthiazol-4-yl)methyl]-3-methylureido]-3-methylbutanoate.

q Thiogalol-3-ylmethyl (2S,3S,5S)-3-hydroxy-5-[(2-[(2-hydroxypropan-2-yl)thiazol-4-yl)methyl]-3-methylureido]acetalamido]-1,6-diphenylhexan-2-ylcarbamate.

r Thiogalol-3-ylmethyl (2S,3S,5S)-3-hydroxy-5-[(S)-4-isopropyl-2,5-dioximidazolidin-1-yl]-1,6-diphenylhexan-2-ylcarbamate.

s Thiogalol-3-ylmethyl (2S,3S,5S)-3-hydroxy-5-[(S)-2-ethoxycarbonylamino]carboxylamino]-3-methylbutanamido]-3-hydroxy-1,6-diphenylhexan-2-ylcarbamate.

t Thiogalol-3-ylmethyl (2S,3S,5S)-3-hydroxy-5-[(S)-2-ethoxycarbonylamino]carboxylamino]-3-methylbutanamido]-3-hydroxy-1,6-diphenylhexan-2-ylcarbamate.

u Thiogalol-3-ylmethyl (2S,3S,5S)-4-benzyl-5-[(S)-2-[(2-isopropylthiazol-4-yl)methyl]-3-methylureido]oxo]oxazolidine-3-carboxylate.

v Thiogalol-3-ylmethyl (2S,3S,5S)-5-[(S)-2-[(2-ethylthiazol-4-yl)methyl]-3-methylureido]oxo]oxazolidine-3-carboxylate.

w Thiogalol-3-ylmethyl (2S,3S,5S)-5-[(S)-2-[(2-ethylthiazol-4-yl)methyl]-3-methylureido]-3-hydroxy-1,6-diphenylhexan-2-ylcarbamate.

x Total ritonavir impurities, specified and unspecified

y A single peak with a relative retention value of 0.44 should be reported as the ethyl carbamate analog due to possible coelution with ritonavir hydroperoxide impurity.

z Thiaolol-3-ylmethyl (2S,3S,5S)-3-hydroxy-5-[(2-[(2-hydroxypropan-2-yl)thiazol-4-yl)methyl]-3-methylureido]acetalamido]-1,6-diphenylhexan-2-ylcarbamate.

To identify the lopinavir impurities, determine the relative retention value from the 215-nm chromatogram relative to ritonavir (see Table 2). Compare the 215-nm chromatogram to the 240-nm chromatogram. Any impurity assigned as a ritonavir impurity at 240 nm that is also observed at 215 nm is discounted. Unspecified ritonavir impurities are assigned according to the algorithm outlined in Table 3.
Not official text. Please refer to the currently official version of the applicable USP–NF or FCC standard for compliance purposes.

The page contains a table listing various compounds along with their acceptance criteria. The table is titled “Table 2” and includes columns for Name, Relative Retention Value (r), Acceptance Criteria (NMT %), and Acceptance (as a percentage).

The table entries include:
- **Lopinavir aminoalcohol**
- **Lopinavir N-formylaminoalcohol**
- **Lopinavir N-acetylaminoalcohol**
- **Lopinavir 2,4-dimethylphenoxacetic amide**
- **Lopinavir N-formylphenoxyacetamide**
- **Lopinavir N-acetylphenoxyacetamide**
- **Lopinavir oxazine**
- **Lopinavir 2-epimer**
- **Lopinavir 4-epimer**
- **Isolopinavir**
- **Lopinavir 2,4-dimethylphenoxy isomer**
- **Lopinavir D-valine diastereomer**
- **Lopinavir (2S,4R) diastereomer**
- **Lopinavir 2-epimer**
- **Any unspecified lopinavir impurity**

The acceptance criteria vary across these compounds, indicating different tolerances for the impurities. For instance, some compounds have a specific range of acceptance criteria, while others have a fixed value.

**Table 3**

This table lists unspecified impurities observed at different wavelengths, with columns for Wavelength (nm), Unspecified Impurity Observed, and Peak assigned to.

The wavelengths include 240 nm and 215 nm. The unspecified impurities observed are associated with the peaks assigned to Ritonavir and Lopinavir.

**SPECIFIC TESTS**

- **Alcohol Determination (611)**
  - **Internal standard solution**: Transfer 10.0 mL of butyl alcohol to a 200-mL volumetric flask and dilute with methanol to volume.
  - **Internal standard identification solution**: Dilute 5.0 mL of Internal standard solution with methanol to 100 mL.
  - **Standard stock solution**: 4.0% (v/v) of dehydrated alcohol in methanol.
  - **Standard solution**: 0.4% (v/v) of dehydrated alcohol prepared as follows. Transfer 10.0 mL of Standard stock solution and 5.0 mL of the Internal standard solution to a 100-mL volumetric flask, and dilute with methanol to volume.

Published on March 26, 2020
Sample stock solution: Transfer 5.0 mL of Oral Solution to a 50-mL volumetric flask with the aid of several portions of methanol, and dilute with methanol to volume.

Sample solution: Transfer 10.0 mL of Sample stock solution and 5.0 mL of the Internal standard solution to a 100-mL volumetric flask, and dilute with methanol to volume.

Chromatographic system
(See Chromatography (621), System Suitability.)

Mode: GC
Detector: Flame ionization
Column: 0.53-mm × 30-m fused silica capillary; coated with a 1-µm film of liquid phase G16

Temperatures
Injection port: 185°
Detector: 220°

Column: See Table 4.

### Table 4

<table>
<thead>
<tr>
<th>Initial Temperature (°)</th>
<th>Temperature Ramp (°/min)</th>
<th>Final Temperature (°)</th>
<th>Hold Time at Final Temperature (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>0</td>
<td>40</td>
<td>5</td>
</tr>
<tr>
<td>40</td>
<td>10</td>
<td>145</td>
<td>6</td>
</tr>
<tr>
<td>145</td>
<td>20</td>
<td>200</td>
<td>9.75</td>
</tr>
</tbody>
</table>

Carrier gas: Helium
Flow rate: 4.5 mL/min
Makeup gas flow: 30 mL/min
Injection volume: 1 µL
Injection type: Split ratio 4:1

System suitability
Sample: Standard solution
Suitability requirements
- Tailing factor: 0.8–1.2 for the alcohol peak
- Relative standard deviation: NMT 3.0% for the peak area ratio of alcohol to the internal standard

Analysis
Samples: Internal standard identification solution, Standard solution, and Sample solution

Calculate the percentage of the labeled amount of alcohol in the portion of Oral Solution taken:

\[
\text{Result} = \left( \frac{R_S}{R_U} \right) \times \left( \frac{C_U}{C_S} \right) \times D \times 100
\]

- \( R_U \) = peak response ratio of alcohol to butyl alcohol from the Sample solution
- \( R_S \) = peak response ratio of alcohol to butyl alcohol from the Standard solution
- \( C_S \) = concentration of dehydrated alcohol in the Standard solution (% v/v)
- \( C_U \) = nominal concentration of alcohol in the Oral Solution (% v/v)
- \( D \) = dilution factor used to prepare the Sample solution

Acceptance criteria: 85.0%–115.0% of the labeled amount of alcohol (C\(_{12}\)H\(_{23}\)O).

- **MICROBIAL ENUMERATION TESTS (61) and TESTS FOR SPECIFIED MICROORGANISMS (62):** The total aerobic microbial count does not exceed 10² cfu/mL.

### ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light. Store at 2°–8°.
- **USP REFERENCE STANDARDS (11)**
  - USP Lopinavir RS
  - USP Ritonavir RS

---

**Memantine Hydrochloride**

C\(_{16}\)H\(_{22}\)N\cdot\)HCl: 215.76

Tricyclo[3.3.1.1\(^3,7\)]decan-1-amine, 3,5-dimethyl-, hydrochloride;
1-Amino-3,5-dimethyladamantane hydrochloride [41100-52-1].

**DEFINITION**

Memantine Hydrochloride contains NLT 98.0% and NMT 102.0% of memantine hydrochloride (C\(_{16}\)H\(_{22}\)N\cdot\)HCl), calculated on the anhydrous basis.

**IDENTIFICATION**

**Change to read:**

- **A. SPECTROSCOPIC IDENTIFICATION TESTS** (197), Infrared Spectroscopy: 197\(^X\) (CN 1-May-2020)
- **B.** The retention time of the major peak of the Sample solution corresponds to that of the Standard solution, as obtained in the Assay.
- **C. IDENTIFICATION TESTS—GENERAL, Chloride** (191): Meets the requirements

**ASSAY**

**PROCEDURE**

Internal standard solution: 4.0 mg/mL of adamantane in n-hexane

Standard solution: 4.0 mg/mL of USP Memantine Hydrochloride RS in Internal standard solution prepared as follows. Transfer 100 mg of USP Memantine Hydrochloride RS to a 50-mL centrifuge tube. Add 15 mL of 1 N sodium hydroxide, and mix. Add 25 mL of Internal standard solution, and shake for 15 min. Allow the layers to separate, and filter a portion of the top hexane layer through anhydrous sodium sulfate. Use the clear filtrate.

Sample solution: 4.0 mg/mL of Memantine Hydrochloride in Internal standard solution prepared as follows. Transfer 100 mg of Memantine Hydrochloride to a 50-mL centrifuge tube. Add 15 mL of 1 N sodium hydroxide, and mix. Add 25 mL of Internal standard solution, and shake for 15 min. Allow the layers to separate, and filter a portion of the top hexane layer through anhydrous sodium sulfate. Use the clear filtrate.

Chromatographic system
(See Chromatography (621), System Suitability.)

Mode: GC
Detector: Flame ionization
Column: 50-m × 0.32-mm; 0.52-µm packing G27

Temperatures
Injection port: 220°
Detector: 300°

Column: See Table 1.

### Table 1

<table>
<thead>
<tr>
<th>Initial Temperature (°)</th>
<th>Temperature Ramp (°/min)</th>
<th>Final Temperature (°)</th>
<th>Hold Time at Final Temperature (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>5</td>
<td>145</td>
<td>0</td>
</tr>
<tr>
<td>145</td>
<td>10</td>
<td>250</td>
<td>20</td>
</tr>
</tbody>
</table>

Carrier gas: Helium
Flow rate: 4.0 ± 0.4 mL/min
**Injection volume:** 1 μL  
**Injection type:** Split ratio, 1:50  

**System suitability**  
**Sample:** Standard solution  
**Suitability requirements**  
Tailing factor: NMT 2.0 each for memantine and adamantane  
Relative standard deviation: NMT 2.0% for the ratio of the peak areas of adamantane and memantine  

**Analysis**  
**Samples:** Standard solution and Sample solution  
Calculate the percentage of memantine hydrochloride (C₈H₁₃N₂H₂O) in the portion of Memantine Hydrochloride taken:  

\[ \text{Result} = (R_u / R_S) \times (C_u / C_S) \times 100 \]

\( R_u \) = peak response ratio of memantine to the internal standard from the Sample solution  
\( R_S \) = peak response ratio of memantine to the internal standard from the Standard solution  
\( C_u \) = concentration of USP Memantine Hydrochloride RS in the Standard solution (mg/mL)  
\( C_S \) = concentration of Memantine Hydrochloride in the Sample solution (mg/mL)  

**Acceptance criteria:** 98.0%–102.0% on the anhydrous basis  

**IMPURITIES**  
- **Residue on ignition (281):** NMT 0.1%  
- **Organic impurities**  
  **Standard stock solution A:** 2.5 mg/mL each of USP Memantine Related Compound A RS, USP Memantine Related Compound B RS, USP Memantine Related Compound C RS, USP Memantine Related Compound D RS, and USP Memantine Related Compound E RS in n-hexane  
  **Standard stock solution B:** 2.5 mg/mL of USP Memantine Hydrochloride RS prepared as follows. To the flask containing a weighed amount of USP Memantine Hydrochloride RS, add 5.0 N sodium hydroxide to fill 20% of the final volume. Shake for 10 min, and transfer the contents to a separator. Allow the layers to separate, filter a portion of the top hexane layer, dry the organic layer by swirling with anhydrous sodium sulfate, and allow to stand for a few min to ensure all the remaining water has been removed. Use the clear filtrate.  

**System suitability solution:** 25 μg/mL each of USP Memantine Related Compound A RS, USP Memantine Related Compound B RS, USP Memantine Related Compound C RS, USP Memantine Related Compound D RS, and USP Memantine Related Compound E RS from Standard stock solution A in Standard stock solution B. The concentration of USP Memantine Hydrochloride RS is 2.5 mg/mL.  

**Standard solution:** 25 μg/mL each of USP Memantine Related Compound A RS, USP Memantine Related Compound B RS, USP Memantine Related Compound C RS, USP Memantine Related Compound D RS, USP Memantine Related Compound E RS, and USP Memantine Hydrochloride RS from Standard stock solution A and Standard stock solution B, respectively, in n-hexane  

**Sample solution:** 25 mg/mL of Memantine Hydrochloride prepared as follows. Transfer the weighed amount of Memantine Hydrochloride to a suitable volumetric flask. Add 5.0 N sodium hydroxide to fill 30% of the final volume and n-hexane to fill 40% of the final volume. Shake for 10 min, and transfer the contents to a separator. Allow the layers to separate, filter a portion of the top hexane layer, dry the organic layer by swirling with anhydrous sodium sulfate, and allow to stand for a few min to ensure all the remaining water has been removed. Use the clear filtrate.  

**Chromatographic system:** Proceed as directed in the Assay.  
**System suitability**  
**Samples:** System suitability solution and Standard solution  
**Suitability requirements**  
Resolution: NLT 6.0 between memantine and memantine related compound B; NLT 2.0 between memantine related compound B and memantine related compound C, System suitability solution  
Tailing factor: NMT 2.0 for memantine, Standard solution  
Relative standard deviation: NMT 10.0% for memantine, Standard solution  

**Analysis**  
**Samples:** Standard solution and Sample solution  
[NOTE— Ignore the peaks at the relative retention times 0.11, 0.12, 0.13, 0.18, and 0.26 with respect to the memantine peak, as they correspond to residual solvents.]  
Calculate the percentage of each of memantine related compounds A, B, C, D, and E in the portion of Memantine Hydrochloride taken:  

\[ \text{Result} = (r_u / r_S) \times (C_u / C_S) \times 100 \]

\( r_u \) = peak response of any other impurity from the Sample solution  
\( r_S \) = peak response of the corresponding USP Memantine Related Compound RS from the Standard solution  
\( C_u \) = concentration of Memantine Hydrochloride in the Sample solution (mg/mL)  
\( C_S \) = concentration of Memantine Hydrochloride RS in the Standard solution (mg/mL)  

Calculate the percentage of any other impurity in the portion of Memantine Hydrochloride taken:  

\[ \text{Result} = (r_u / r_S) \times (C_u / C_S) \times 100 \]

<table>
<thead>
<tr>
<th>Name</th>
<th>Relative Retention Time</th>
<th>Acceptance Criteria, NMT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Memantine related compound A</td>
<td>0.77</td>
<td>0.15</td>
</tr>
<tr>
<td>Memantine</td>
<td>1.0</td>
<td>—</td>
</tr>
</tbody>
</table>

**Table 2**
Memantine Hydrochloride Tablets

**DEFINITION**
Memantine Hydrochloride Tablets contain an amount of memantine hydrochloride equivalent to NLT 90.0% and NMT 110.0% of the labeled amount of memantine hydrochloride (C$_{12}$H$_{19}$N·HCl).

**IDENTIFICATION**

**Change to read:**

- **A. Spectroscopic Identification Tests** (197), Infrared Spectroscopy: 197K
  *Analytical range: 4000–400 cm$^{-1}$*
  *Standard: 6.7 mg/mL of USP Memantine Hydrochloride RS in dichloromethane. Shake for 10 min, and pass through a suitable filter. Evaporate the solvent at room temperature. Collect the residue powder, and dry at 60° for 15 min. Prepare an approximate 1% (w/w) dispersion of the sample in potassium bromide.**
  *Sample: 6.7 mg/mL of memantine hydrochloride in dichloromethane from NLT 20 crushed Tablets. Shake for 10 min, and centrifuge for 10 min. Pass the supernatant through a suitable filter. Evaporate the solvent at room temperature. Collect the residue powder, and dry at 60° for 15 min. Prepare an approximate 1% (w/w) dispersion of the sample in potassium bromide.**

**ASSAY**

- **Procedure**
  *Solution A: 200 mg/mL of sodium hydroxide in water*
  *Internal standard solution: 25 µg/mL of USP Amantadine Hydrochloride RS in water*
  *Standard stock solution: 25 µg/mL of USP Memantine Hydrochloride RS prepared as follows. Weigh a suitable quantity of the Standard into a volumetric flask. Add methanol to fill 40% of the final flask volume, and sonicate. Dilute with water to volume.*
  *Sample stock solution: Nominally 20 µg/mL of memantine hydrochloride prepared as follows. Transfer a suitable number of Tablets to a volumetric flask to obtain a 0.1 mg/mL memantine hydrochloride solution. Add methanol to fill 40% of the final flask volume, and sonicate for 30 min with intermittent shaking. Add water to fill 40% of the final flask volume, and sonicate for 30 min with intermittent shaking. Dilute with water to volume, and centrifuge a portion for 10 min. Pipet a suitable volume of the clear centrifugate into a volumetric flask, and dilute with water to volume.*
  *Blank: To 5.0 mL of 80 µL/mL of methanol in water add 2 mL of Solution A, and mix on a vortex mixer for 1 min. Add 4.0 mL of toluene, and mix on a vortex mixer for 5 min.*
  *Table 1*

<table>
<thead>
<tr>
<th>Initial Temperature (°)</th>
<th>Temperature Ramp (°C/min)</th>
<th>Final Temperature (°)</th>
<th>Hold Time at Final Temperature (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
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<td>50</td>
<td>2</td>
</tr>
<tr>
<td>50</td>
<td>20</td>
<td>140</td>
<td>0</td>
</tr>
<tr>
<td>140</td>
<td>30</td>
<td>200</td>
<td>5</td>
</tr>
</tbody>
</table>

**Chromatographic system**
(See Chromatography (621), System Suitability.)
*Mode: GC*
*Detector: Flame ionization*
*Column: 30-m × 0.32-mm; 0.25-µm packing G27*
*Temperatures*
*Injection port: 210°*
*Detector: 300°*
*Oven: See Table 1.*

**Table 1**

- **Carrier gas:** Helium
- **Flow rate:** 34.8 psi
- **Injection volume:** 4 µL
- **Injection type:** Split ratio, 1:10
System suitability
**Sample:** Standard solution
[Note—The relative retention times for amantadine and memantine are 0.97 and 1.0, respectively.]

**Suitability requirements**
- Resolution: NLT 2.0 between amantadine and memantine
- Tailing factor: NMT 2.5 for amantadine; NMT 2.0 for memantine
- Relative standard deviation: NMT 2.0% for the ratio of the peak areas of amantadine and memantine

**Analysis**
**Samples:** Standard solution, Sample solution, and Blank
Calculate the percentage of the labeled amount of memantine hydrochloride (C<sub>12</sub>H<sub>22</sub>N·HCl) in the portion of Tablets taken:

\[
\text{Result} = \left( \frac{R_U}{R_S} \right) \times \left( \frac{C_S}{C_U} \right) \times 100
\]

- \( R_U \) = peak area ratio of memantine to amantadine from the Sample solution
- \( R_S \) = peak area ratio of memantine to amantadine from the Standard solution
- \( C_S \) = concentration of USP Memantine Hydrochloride RS in the Standard solution (µg/mL)
- \( C_U \) = nominal concentration of memantine hydrochloride in the Sample solution (µg/mL)

**Acceptance criteria:** 90.0%–110.0%

**PERFORMANCE TESTS**
- **Dissolution** (711)
  - **Medium:** 0.1 N hydrochloric acid with sodium chloride (2 g/L of sodium chloride in water), adjusted with hydrochloric acid to a pH of 1.2; 900 mL
  - **Apparatus 1:** 100 rpm
  - **Time:** 30 min
  - **Standard stock solution:** \((L/900)\) mg/mL of USP Memantine Hydrochloride RS in Medium, where \(L\) is the label claim in mg/Tablet
  - **Internal standard solution:** 28 µg/mL of USP Amantadine Hydrochloride RS in Medium
  - **Standard solution**
    - **For Tablets labeled to contain 5 mg:** Transfer 5 mL of the Standard stock solution to a test tube, add 1 mL of the Internal standard solution and 2 mL of 5 N sodium hydroxide, and mix for 1 min. Add 3 mL of toluene, and mix for 2 min. Use the toluene layer.
    - **For Tablets labeled to contain 10 mg:** Transfer 5 mL of the Standard stock solution to a test tube, add 2 mL of the Internal standard solution and 2 mL of 5 N sodium hydroxide, and mix for 1 min. Add 3 mL of toluene, and mix for 2 min. Use the toluene layer.
  - **Sample solution:** Pass a portion of the solution under test through a suitable filter.
    - **For Tablets labeled to contain 5 mg:** Transfer 5 mL of the filtrate to a test tube, add 1 mL of the Internal standard solution and 2 mL of 5 N sodium hydroxide, and mix for 1 min. Add 3 mL of toluene, and mix for 2 min. Use the toluene layer.
    - **For Tablets labeled to contain 10 mg:** Transfer 5 mL of the filtrate to a test tube, add 2 mL of the Internal standard solution and 2 mL of 5 N sodium hydroxide, and mix for 1 min. Add 3 mL of toluene, and mix for 2 min. Use the toluene layer.
  - **Chromatographic system**
    - **Mode:** GC, splitless
    - **Detector:** Flame ionization

**Column:** 30-m × 0.32-mm; 0.25-µm packing G27
**Flow rate:** 34.8 psi
**Temperatures**
- **Injection port:** 210°
- **Detector:** 300°
- **Oven:** See Table 2.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Temperature Ramp (°C/min)</th>
<th>Final Temperature (°C)</th>
<th>Hold Time at Final Temperature (min)</th>
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<tr>
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</tr>
<tr>
<td>140</td>
<td>30</td>
<td>200</td>
<td>5</td>
</tr>
</tbody>
</table>

**Carrier gas:** Helium
**Injection volume:** 4 µL

**System suitability**
- **Sample:** Standard solution

**Suitability requirements**
- **Resolution:** NLT 2.0 between amantadine and memantine
- **Tailing factor:** NMT 2.0 each for amantadine and memantine
- **Relative standard deviation:** NMT 2.0% for the ratio of memantine to amantadine peaks

**Analysis**
- **Samples:** Standard solution and Sample solution
  - Calculate the percentage of the labeled amount of memantine hydrochloride (C<sub>12</sub>H<sub>22</sub>N·HCl) dissolved:
    \[
    \text{Result} = \left( \frac{R_U}{R_S} \right) \times \left( \frac{C_S}{L} \right) \times V \times 100
    \]
    - \( R_U \) = peak area ratio of memantine to amantadine from the Sample solution
    - \( R_S \) = peak area ratio of memantine to amantadine from the Standard solution
    - \( C_S \) = concentration of USP Memantine Hydrochloride RS in the Standard stock solution (mg/mL)
    - \( L \) = label claim (mg/Tablet)
    - \( V \) = volume of Medium, 900 mL

**Tolerances:** NLT 80% (Q) of the labeled amount of memantine hydrochloride (C<sub>12</sub>H<sub>22</sub>N·HCl) is dissolved.

**Uniformity of Dosage Units** (905): Meet the requirements

**Impurities**
- **Limit of Memantine–Lactose Adduct**
  - [Note—Perform this test if lactose is present in the formulation.]

**Solution A:** 40 mg/mL of sodium hydroxide in water
**Buffer:** Dissolve 3.3 g of monobasic potassium phosphate and 2.3 g of sodium 1-octane sulfonate in 1 L of water. Adjust with Solution A to a pH of 6.1.
**Mobile phase:** Acetonitrile, methanol, and Buffer (26:4:70)
**Standard solution:** 0.2 mg/mL of USP Memantine Hydrochloride RS in Mobile phase
**Sample solution:** Nominally 10 mg/mL of memantine hydrochloride from NLT 25 crushed Tablets, prepared as follows. Transfer an amount of powder equivalent to 100 mg of memantine hydrochloride to a 20-mL volumetric flask. Add 10 mL of Mobile phase, and sonicate for 30 min. Centrifuge, and pass a portion of the centrifugate through a suitable filter of 0.45-µm pore size.
Chromatographic system
(See Chromatography (621), System Suitability.)
Mode: LC
Detector: Refractive index
Column: 4.6-mm × 15-cm; 5-µm packing L1
Temperatures
Column: 40°
Detector: 35°
Flow rate: 1.3 mL/min
Injection volume: 50 µL
Run time: 1.3 times the retention time of the
memantine peak
System suitability
Sample: Standard solution
Suitability requirements
Tailing factor: NMT 2.0
Relative standard deviation: NMT 10.0%
Analysis
Samples: Standard solution and Sample solution
Calculate the percentage of the memantine–lactose adduct in the portion of Tablets taken:
\[
\text{Result} = \left( \frac{r_s}{r_0} \right) \times \left( \frac{C_s / C_0}{1/F} \right) \times 100
\]
\[r_0 = \text{peak response of the memantine–lactose adduct from the Sample solution}
\]
\[r_s = \text{peak response of memantine from the Standard solution}
\]
\[C_s = \text{concentration of USP Memantine Hydrochloride RS in the Standard solution (mg/mL)}
\]
\[C_0 = \text{nominal concentration of memantine hydrochloride in the Sample solution (mg/mL)}
\]
\[F = \text{relative response factor of the memantine–lactose adduct (see Table 3)}
\]
Acceptance criteria: See Table 3.
Disregard all peaks other than the memantine–lactose adduct peak.

<table>
<thead>
<tr>
<th>Name</th>
<th>Relative Retention Time</th>
<th>Relative Response Factor</th>
<th>Acceptance Criteria, NMT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Memantine–lactose adduct</td>
<td>0.41</td>
<td>0.53</td>
<td>1.4</td>
</tr>
<tr>
<td>Memantine</td>
<td>1.0</td>
<td>1.0</td>
<td>—</td>
</tr>
</tbody>
</table>

• ORGANIC IMPURITIES
Solution A: 1 N sodium hydroxide
System suitability stock solution A: 0.5 mg/mL each of USP Memantine Related Compound B RS, USP Memantine Related Compound C RS, USP Memantine Related Compound D RS, and USP Memantine Related Compound E RS in n-hexane
System suitability stock solution B: Transfer 75 mg of USP Memantine Hydrochloride RS into a suitable container, add 9 mL of 1.0 N sodium hydroxide and 6 mL of n-hexane, and mix for 10 min.
System suitability solution: Pipet 4.0 mL of the n-hexane layer from System suitability stock solution B into a 10-mL volumetric flask. Add 0.5 mL of System suitability stock solution A, and dilute with n-hexane to volume.
Standard stock solution: 1.3 mg/mL of USP Memantine Hydrochloride RS in n-hexane prepared as follows. Weigh a suitable quantity of the Standard into a volumetric flask. Add Solution A to fill 30% of the final flask volume, and mix for 5 min. Add n-hexane to fill 40% of the final flask volume, and shake for 10 min. Transfer the contents of the flask into a separator. Allow the layers to separate, and filter a portion of the top n-hexane layer through anhydrous sodium sulfate. Use the clear solution.
Standard solution: Pipet 2.0 mL of the clear solution from the Standard stock solution into a 100-mL volumetric flask, and dilute with n-hexane to volume.
Sample solution: Nominally 5 mg/mL of memantine hydrochloride in n-hexane from NLT 20 crushed Tablets, prepared as follows. Transfer a weighed amount of powder equivalent to 100 mg of memantine hydrochloride to a suitable volumetric flask. Add Solution A to fill 15% of the final flask volume. Shake to disperse the material, and then shake for 5 min. Sonicate for 5 min with intermittent shaking. Add n-hexane to fill 20% of the final flask volume, and shake for 10 min. Transfer the contents into a separator. Allow the layers to separate, and filter a portion of the top hexane layer through anhydrous sodium sulfate. Use the clear solution.

Table 3

<table>
<thead>
<tr>
<th>Name</th>
<th>Relative Retention Time</th>
<th>Relative Response Factor</th>
<th>Acceptance Criteria, NMT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Memantine–lactose adduct</td>
<td>0.41</td>
<td>0.53</td>
<td>1.4</td>
</tr>
<tr>
<td>Memantine</td>
<td>1.0</td>
<td>1.0</td>
<td>—</td>
</tr>
</tbody>
</table>

Carrier gas: Helium
Flow rate: 4.0 ± 0.2 mL/min
Injection volume: 3 µL
Injection type: Split ratio, 1:20
System suitability
Samples: System suitability solution and Standard solution
[NOTE—See Table 5 for the relative retention times.]
Suitability requirements
Resolution: NLT 2.0 between memantine and memantine related compound B; NLT 2.0 between memantine related compound B and memantine related compound C; System suitability solution
Tailing factor: NMT 2.0, Standard solution
Relative standard deviation: NMT 10.0%, Standard solution
Analysis
Samples: Standard solution and Sample solution
Calculate the percentage of memantine related compound E or any individual degradation product in the portion of Tablets taken:
\[
\text{Result} = \left( \frac{r_s}{r_0} \right) \times \left( \frac{C_s / C_0}{1/F} \right) \times 100
\]
\[r_0 = \text{peak response of memantine related compound E or any individual degradation product from the Sample solution}
\]
\[r_s = \text{peak response of memantine hydrochloride from the Standard solution}
\]

Table 4

<table>
<thead>
<tr>
<th>Initial Temperature (°)</th>
<th>Temperature Ramp (°/min)</th>
<th>Final Temperature (°)</th>
<th>Hold Time at Final Temperature (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>0</td>
<td>50</td>
<td>2</td>
</tr>
<tr>
<td>50</td>
<td>5</td>
<td>145</td>
<td>0</td>
</tr>
<tr>
<td>145</td>
<td>10</td>
<td>250</td>
<td>20</td>
</tr>
</tbody>
</table>
$$C_s = \text{concentration of USP Memantine Hydrochloride RS in the Standard solution (mg/mL)}$$

$$C_0 = \text{nominal concentration of memantine hydrochloride in the Sample solution (mg/mL)}$$

**Acceptance criteria:** See Table 5.

<table>
<thead>
<tr>
<th>Name</th>
<th>Relative Retention Time</th>
<th>Acceptance Criteria, NMT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Memantine related compound A*</td>
<td>0.77</td>
<td>—</td>
</tr>
<tr>
<td>Memantine</td>
<td>1.0</td>
<td>—</td>
</tr>
<tr>
<td>Memantine related compound B*</td>
<td>1.03</td>
<td>—</td>
</tr>
<tr>
<td>Memantine related compound C*</td>
<td>1.1</td>
<td>—</td>
</tr>
<tr>
<td>Memantine related compound D*</td>
<td>1.2</td>
<td>—</td>
</tr>
<tr>
<td>Memantine related compound E</td>
<td>1.4</td>
<td>0.3</td>
</tr>
<tr>
<td>Any individual unspecified degradation product</td>
<td>—</td>
<td>0.20</td>
</tr>
<tr>
<td>Total impurities*</td>
<td>—</td>
<td>0.5</td>
</tr>
</tbody>
</table>

*Process impurities controlled in the drug substance and are included for identification only. Not reported for the drug product and not included in the total impurities.

**Packaging and storage**—Preserve in well-closed containers. Avoid moisture and excessive heat.

**Labeling**—Label it to indicate that it is for veterinary use only. Label it also to state that it is for manufacturing, processing, or repackaging.

**USP Reference standards** (11)—

USP Monensin Sodium RS
USP Narasin RS

**Identification**—The chromatogram of the Assay preparation obtained as directed in the Assay exhibits a major peak for monensin A and a minor peak for monensin B, the retention times of which correspond to those exhibited in the chromatogram of the Standard preparation, obtained as directed in the Assay.

**Loss on drying** (731)—Dry it in vacuum at 60°C for 2 hours: it loses not more than 10% of its weight.

**Content of monensin A and B activity**—Using the results of the calculations in the Assay, calculate the percentage of monensin A activity in the Monensin under test by the formula:

$$\frac{100A}{P}$$

in which A is the potency, in µg per mg, of monensin A in the Monensin under test, as determined in the Assay, and P is the potency, in µg of monensin, in each mg of the Monensin under test, as determined in the Assay, not less than 90% is found. Calculate the percentage of monensin A activity plus monensin B activity in the Monensin under test by the formula:

$$\frac{100(A + B)}{P}$$

in which B is the potency, in µg per mg, of monensin B in the Monensin under test, as determined in the Assay, and the other terms are as defined above: not less than 95% is found.

**Assay**—

**Mobile phase**—Prepare a filtered and degassed mixture of methanol, water, and glacial acetic acid (94: 6: 0.1). Make adjustments if necessary (see System Suitability under Chromatography (621)).

**Neutralized methanol**—Add 1 g of sodium bicarbonate to 4 liters of methanol, mix, and filter.

**Diluent**—Prepare a mixture of methanol and water (9:1).
Derivatizing reagent—Dissolve 3 g of vanillin in a mixture of 95 mL of methanol and 2 mL of sulfuric acid. [Caution—To avoid splattering, add the sulfuric acid carefully and slowly with a pipet; do not pour. Allow the mixture of methanol and sulfuric acid to cool before adding vanillin.]

Standard preparation—Dissolve an accurately weighed quantity of USP Monensin Sodium RS quantitatively in methanol to obtain a solution containing the equivalent of 1000 µg of monensin per mL. Dilute an accurately measured volume of this stock solution quantitatively with Diluent to obtain a solution containing 20.0 µg of monensin per mL.

Assay preparation—Transfer about 500 mg of Monensin, accurately weighed, to a 250-mL flask, add 200.0 mL of Diluent, and shake by mechanical means for 1 hour. Allow the solids to settle, and dilute an accurately measured volume of the supernatant quantitatively with Diluent to obtain a solution containing about 20 µg of monensin per mL.

Resolution solution—Prepare a solution in Neutralized methanol containing about 1 mg of USP Monensin Sodium RS and 3 mg of USP Narasin RS per mL. Transfer 2 mL of this solution to a 200-mL volumetric flask, dilute with Diluent to volume, and mix.

Chromatographic system (see Chromatography (621))—The liquid chromatograph is equipped with a 4.6-mm × 25-cm column that contains packing L1 and the outlet of which is attached to a detector set at 520 nm. The Mobile phase and the Derivatizing reagent flow at the rate of about 0.7 mL per minute. Chromatograph the Resolution solution, and record the peak responses as directed under Procedure: the relative retention times are about 0.9 for monensin B, 1.0 for monensin A, 1.3 for narasin A, and 1.5 for narasin I, the resolution, \( R_t \), between the monensin B peak and the monensin A peak is not less than 1.25, and between the monensin A peak and the narasin A peak is not less than 3.5. Chromatograph the Standard preparation, and record the peak responses as directed under Procedure: the tailing factor is not more than 1.4, and the relative standard deviation for replicate injections is not more than 2.0%. [NOTE—After use, flush the system with methanol.]

Procedure—[NOTE—Use peak areas where peak responses are indicated.] Separately inject equal volumes (about 200 µL) of the Standard preparation and the Assay preparation into the chromatograph, record the chromatograms, and measure the responses for the major peaks, including a peak for monensin C/D, if present, at a retention time of about 1.1 relative to that of the main monensin A peak in the chromatogram obtained from the Assay preparation. Calculate the quantity, in µg, of monensin A in each mg of the Monensin taken by the formula:

\[
(CFD/100,000W)(r_u/r_3)
\]

in which \( C \) is the concentration, in µg per mL, of monensin activity in the Standard preparation, based on the quantity of USP Monensin Sodium RS taken, its designated potency, in µg per mg, and the extent of dilution, \( F \) is the designated percentage of monensin A in USP Monensin Sodium RS, \( D \) is the dilution factor used in preparing the Assay preparation, \( W \) is the quantity, in g, of Monensin taken to prepare the Assay preparation, and \( r_u \) and \( r_3 \) are the monensin A peak responses obtained from the Assay preparation and the Standard preparation, respectively. Calculate the quantity, in µg, of monensin B in each mg of the Monensin taken by the same formula, except that \( r_B \) is the monensin B peak response obtained from the Assay preparation and \( r_l \) is the monensin A peak response obtained from the Standard preparation.

Calculate the quantity, in µg, of monensin C/D in each mg of the Monensin taken by the same formula, except that \( r_c \) is the monensin C/D peak response obtained from the Assay preparation. Calculate the potency, in µg of monensin, in each mg of the Monensin taken by the formula:

\[
A + 0.288 + 1.5C/D
\]

in which \( A \) is the quantity, in µg, of monensin A in each mg of the Monensin taken, as calculated above, and \( B \) is the quantity, in µg, of monensin B in each mg of the Monensin taken, and \( C/D \) is the quantity, in µg, of monensin C/D in each mg of Monensin taken, as calculated above.

Monensin Sodium

<table>
<thead>
<tr>
<th>Compound</th>
<th>Formula</th>
<th>Potency (µg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C56H43NaO11</td>
<td>monensin A sodium</td>
<td>692.85</td>
</tr>
<tr>
<td>C56H43NaO11</td>
<td>monensin B sodium</td>
<td>678.83</td>
</tr>
<tr>
<td>C49H40NaO11</td>
<td>monensin C sodium</td>
<td>706.88</td>
</tr>
</tbody>
</table>

Monensin sodium salt.

Stereoisomer of 2-[2-ethyloctahydro-3′-methyl-5′-tettahydro-6-hydroxy-6-(hydroxymethyl)-3,5-dimethyl-2H-pyran-2-yl][2,2′-bifuran-5-yl]-9-hydroxy-β-methoxy-α,γ,2,8-tetramethyl-1,6-dioxaspiro[4.5]decan-7-butanonic acid sodium salt [22373-78-0].

Monensin Sodium has a potency of not less than 800 µg per mg.

Packaging and storage—Preserve in well-closed containers. Avoid moisture and excessive heat.

Labeling—Label it to indicate that it is for veterinary use only. Label it also to state that it is for manufacturing, processing, or repackaging.

USP Reference standards (11)—USP Monensin Sodium RS USP Narasin RS

Identification—The chromatogram of the Assay preparation obtained as directed in the Assay exhibits a major peak for monensin A and a minor peak for monensin B, the retention times of which correspond to those exhibited in the chromatogram of the Standard preparation, as obtained in the Assay.

Loss on drying (731)—Dry it in vacuum at 60° for 3 hours: it loses not more than 4% of its weight.

Content of monensin A and B activity—Using the results of the calculations in the Assay, calculate the percentage of monensin A activity in the Monensin Sodium under test by the formula:

\[
100A/P
\]

in which \( A \) is the potency, in mg per g, of monensin A in the Monensin Sodium under test, as determined in the Assay, and \( P \) is the potency, in mg of monensin, in each g of the Monensin Sodium under test, as determined in the Assay; not less than 90% is found. Calculate the percentage of monensin A activity plus monensin B activity in the Monensin Sodium under test by the formula:

\[
100(A + B)/P
\]

in which \( B \) is the potency, in mg per g, of monensin B in the Monensin Sodium under test, as determined in the Assay, and...
the other terms are as defined above: not less than 95% is found.

Assay—
Mobile phase, Neutralized methanol, Diluent, Derivatizing reagent, Standard preparation, Resolution solution, and Chromatographic system—Proced as directed in the Assay under Monensin.
Assay preparation—Transfer about 100 mg of Monensin Sodium, accurately weighed, to a 100-mL volumetric flask, dissolve in and dilute with methanol to volume. If necessary, to achieve complete dissolution, sonicate for about 1 minute, and mix. Dilute an accurately measured volume of this solution quantitatively with Diluent to obtain a solution containing about 20 µg of monensin per mL.
Procedure—Proced as directed for Procedure in the Assay under Monensin. Calculate the quantity, in mg, of monensin A in each g of the Monensin Sodium taken by the formula:

\[(C/D) \times 100,000 \times (r_U/r_C)\]

in which \(C\) is the concentration, in µg per mL, of monensin activity in the Standard preparation, based on the quantity of USP Monensin Sodium RS taken, its designated potency, in µg per mg, and the extent of dilution; \(F\) is the designated percentage of monensin A in USP Monensin Sodium RS; \(D\) is the dilution factor used in preparing the Assay preparation; \(W\) is the quantity, in g, of Monensin Sodium taken to prepare the Assay preparation; and \(r_U\) and \(r_C\) are the monensin A peak responses obtained from the Assay preparation and the Standard preparation, respectively. Calculate the quantity, in mg, of monensin B in each g of the Monensin Sodium taken by the same formula, except that \(r_U\) is the monensin B peak response obtained from the Assay preparation, and \(r_C\) is the monensin A peak response obtained from the Standard preparation. Calculate the quantity, in mg of monensin, in each g of the Monensin Sodium taken by the formula:

\[A + 0.28B + 1.5C/D\]

in which \(A\) is the quantity, in mg, of monensin A in each g of the Monensin Sodium taken, as calculated above; \(B\) is the quantity, in mg, of monensin B in each g of the Monensin Sodium taken; and \(C/D\) is the quantity, in mg, of monensin \(C/D\) in each g of Monensin Sodium taken, as calculated above.

USP Reference standards (11)—
USP Monensin Sodium RS
USP Narasin RS
Identification—The chromatogram of the Assay preparation obtained as directed in the Assay exhibits a major peak for monensin A and a minor peak for monensin B, the retention times of which correspond to those exhibited in the chromatogram of the Standard preparation, obtained as directed in the Assay.
Loss on drying (731)—Dry it in vacuum at 60° for 2 hours: it loses not more than 10% of its weight.

Content of monensin A and B activity—Using the results of the calculations in the Assay, calculate the percentage of monensin A activity in the Monensin Granulated under test by the formula:

\[100A/P\]

in which \(A\) is the potency, in µg per mg, of monensin A in the Monensin Granulated under test, as determined in the Assay, and \(P\) is the potency, in µg of monensin, in each mg of the Monensin Granulated under test, as determined in the Assay; not less than 90% is found. Calculate the percentage of monensin A activity plus monensin B activity in the Monensin Granulated under test by the formula:

\[100(A + B)/P\]

in which \(B\) is the potency, in µg per mg, of monensin B in the Monensin Granulated under test, as determined in the Assay, and the other terms are as defined above: not less than 95% is found.

Assay—
Mobile phase, Neutralized methanol, Diluent, Derivatizing reagent, Standard preparation, Resolution solution, and Chromatographic system—Proced as directed in the Assay under Monensin.
Assay preparation—Transfer about 5 g of Monensin Granulated, accurately weighed, to a 250-mL flask, add 200.0 mL of Diluent, and shake by mechanical means for 1 hour. Allow the solids to settle, and dilute an accurately measured volume of the supernatant quantitatively with Diluent to obtain a solution containing about 20 µg of monensin per mL.
Procedure—Proced as directed for Procedure in the Assay under Monensin. Calculate the quantity, in mg, of monensin A in each g of the Monensin Granulated taken by the formula:

\[(C/D) \times 100,000 \times (r_U/r_C)\]

in which \(C\) is the concentration, in µg per mL, of monensin activity in the Standard preparation, based on the quantity of USP Monensin Sodium RS taken, its designated potency, in µg per mg, and the extent of dilution, \(F\) is the designated percentage of monensin A in USP Monensin Sodium RS; \(D\) is the dilution factor used in preparing the Assay preparation, \(W\) is the quantity, in g, of Monensin Granulated taken to prepare the Assay preparation, and \(r_U\) and \(r_C\) are the monensin A peak responses obtained from the Assay preparation and the Standard preparation, respectively. Calculate the quantity, in mg, of monensin B in each g of the Monensin Granulated taken by the same formula, except that \(r_U\) is the monensin B peak response obtained from the Assay preparation and \(r_C\) is the monensin A peak response obtained from the Standard preparation. Calculate the quantity, in mg, of monensin \(C/D\) in each g of the Monensin Granulated taken by the same formula, except that \(r_U\) is the monensin C/D peak response obtained from the Assay preparation. Calculate the percentage of monensin A activity plus monensin B activity in the Monensin Granulated under test by the formula:

\[100(A + B)/P\]

in which \(B\) is the potency, in µg per mg, of monensin B in the Monensin Granulated under test, as determined in the Assay, and the other terms are as defined above: not less than 95% is found.

Monensin Granulated

Monensin Granulated contains Monensin mixed with suitable diluents, carriers, and inactive ingredients prepared in a granulated form that is free-flowing and free from aggregates. It may contain added Monensin Sodium. It contains not less than 140 mg of monensin per g.

Packaging and storage—Preserve in well-closed containers. Avoid moisture and excessive heat.
Labeling—Label it to indicate that it is for veterinary use only. Label it also to state that it is for manufacturing, processing, or repackaging.
Monensin Type A Medicated Article

DEﬁnition
Monensin Type A Medicated Article contains Monensin Granulated mixed with suitable diluents and inactive ingredients. It contains the equivalent of NLT 85.0% and NMT 115.0% of the labeled amount of monensin.

Identiﬁcation
• A. The retention times of the major peak for monensin A and minor peak for monensin B of the Sample solution correspond to those of the Standard solution, as obtained in the Assay.

Assay
• Procedure
Mobile phase: Methanol, glacial acetic acid, and water (940:1:60)
Neutralized methanol: Add 1 g of sodium bicarbonate to 4 L of methanol, mix, and ﬁlter.
Diluent: Methanol and water (9:1)
Derivatizing reagent: Dissolve 3 g of vanillin in a mixture of 95 mL of methanol and 2 mL of sulfuric acid.
[CAUTION]—To avoid splattering, add the sulfuric acid carefully and slowly with a pipet; do not pour. Allow the mixture of methanol and sulfuric acid to cool before adding vanillin.

System suitability solution: 1 mg/mL of USP Monensin Sodium RS and 3 mg/mL of USP Narasin RS in Neutralized methanol. Dilute 2 mL of this solution with Diluent to 200 mL.

Standard stock solution: 1000 µg/mL of monensin from USP Monensin Sodium RS in methanol

Standard solution: 20 µg/mL of monensin from Standard stock solution in Diluent

Sample stock solution: Dilute 5 g of Monensin Type A Medicated Article in 200.0 mL of Diluent, and shake by mechanical means for 1 h. Allow the solids to settle.

Sample solution: Nominally 20 µg/mL of monensin, from the clear supernatant of the Sample stock solution, in Diluent

Chromatographic system
(See Chromatography (621), System Suitability.)
Mode: LC
Detector: UV 520 nm
Column: 4.6-mm × 25-cm; packing L1. The column outlet is attached to a tee, the opposing arm is attached to a tube from which is pumped the Derivatizing reagent, and the outlet is connected to a 2-mL postcolumn reaction coil maintained at 98°. The outlet of the reaction coil is connected to the Detector.
Flow rate: 0.7 mL/min for Mobile phase and Derivatizing reagent
Injection volume: 200 µL

Suitability Requirements
Resolution: NLT 1.25 between the monensin B and the monensin A peaks; NLT 3.5 between the monensin A and the narasin A peaks, System suitability solution
Tailing factor: NMT 1.4, Standard solution
Relative standard deviation: NMT 2.0%, Standard solution

[NOTE]—After use, flush the system with methanol.

Analysis
Samples: Standard solution and Sample solution

[NOTE]—Use peak areas where peak responses are indicated.

Calculate the potency, in mg, of monensin in each g of Monensin Type A Medicated Article taken:

\[
\text{Result} = \left( \frac{U_r}{S_r} \right) \times \left( \frac{C_S \times F \times D}{100,000 \times W} \right)
\]

\[A = \text{quantity of monensin A in each g of Monensin Type A Medicated Article taken, as calculated previously (mg)}\]

\[F_d = \text{biopotency conversion factor for monensin A, 1.00}\]

\[B = \text{quantity of monensin B in each g of Monensin Type A Medicated Article taken, as calculated previously (mg)}\]

\[F_b = \text{biopotency conversion factor for monensin B, 0.28}\]

\[C/D = \text{quantity of monensin C/D in each g of Monensin Type A Medicated Article taken, as calculated previously (mg)}\]

\[F_{C/D} = \text{biopotency conversion factor for monensin C/D, 1.50}\]

Acceptance criteria: 85.0%–115.0%

Specific Tests
• Loss on Drying (731)
Analysis: Dry under vacuum at 60° for 2 h.
Acceptance criteria: NMT 10%
Mycophenolic Acid Delayed-Release Tablets

DEFINITION
Mycophenolic Acid Delayed-Release Tablets contain an amount of mycophenolate sodium equivalent to NLT 95.0% and NMT 105.0% of the labeled amount of mycophenolic acid (C$_{17}$H$_{29}$O$_{7}$).

IDENTIFICATION
- **A.** The retention time of the major peak of the Sample solution corresponds to that of the Standard solution, as obtained in the Assay.
- **B.** The UV spectrum of the major peak of the Sample solution corresponds to that of the Standard solution, as obtained in the Assay.

ASSAY
- **PROCEDURE**
  - Protect solutions from light.
  - **Solution A:** Dissolve 21 g of citric acid in a suitable volume of water, add 200 mL of 1 M sodium hydroxide solution, and dilute with water to 1 L.
  - **Buffer:** Solution A and 0.1 M hydrochloric acid (399:601)
  - **Solution B:** Acetonitrile, Buffer, and water (40:15:45)
  - **Solution C:** Acetonitrile and Buffer (85:15)
  - **Mobile phase:** See Table 1. Return to original conditions, and re-equilibrate the system.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Solution B (%)</th>
<th>Solution C (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>18</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>22</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

- **Diluent:** Solution B
- **Standard solution:** 0.385 mg/mL of USP Mycophenolate Sodium RS in Diluent. Stir magnetically for at least 60 min to aid dissolution.
- **Sample stock solution:** Nominally equivalent to 9 mg/mL of mycophenolic acid in Diluent prepared as follows. Transfer NLT 25 Tablets to a volumetric flask and add Diluent to volume. Add a stirring bar and stir vigorously for at least 60 min. Centrifuge a portion of the suspension and use the clear supernatant.
- **Sample solution:** Nominally equivalent to 0.36 mg/mL of mycophenolic acid in Diluent from Sample stock solution

Chromatographic system
(See Chromatography (621), System Suitability.)
Mode: LC
Detector: UV 251 nm or diode array. [Note—Use a diode array detector to perform Identification B.]

<table>
<thead>
<tr>
<th>Column: 4.6-mm × 25-cm; 5-µm packing L7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column temperature: 40°C</td>
</tr>
<tr>
<td>Flow rate: 1 mL/min</td>
</tr>
<tr>
<td>Injection volume: 10 µL</td>
</tr>
</tbody>
</table>

**System suitability**
- **Sample:** Standard solution
- **Suitability requirements**
  - Tailing factor: NMT 2.0
  - Relative standard deviation: NMT 1.0%

**Analysis**
- **Samples:** Standard solution and Sample solution
Calculate the percentage of the labeled amount of mycophenolic acid (C$_{17}$H$_{29}$O$_{7}$) in the portion of Tablets taken:

$$\text{Result} = \left( \frac{r_u}{r_s} \right) \times \left( \frac{C_u}{C_s} \right) \times \left( \frac{M_{r1}}{M_{r2}} \right) \times 100$$

- $r_u$ = peak response of mycophenolate from the Sample solution
- $r_s$ = peak response of mycophenolate from the Standard solution
- $C_u$ = concentration of USP Mycophenolate Sodium RS in the Standard solution (mg/mL)
- $C_s$ = concentration of mycophenolic acid in the Sample solution (mg/mL)
- $M_{r1}$ = molecular weight of mycophenolic acid, 320.34
- $M_{r2}$ = molecular weight of mycophenolate sodium, 342.32

**Acceptance criteria:** 95.0%–105.0%

OTHER TESTS
- **Dissolution** (711)
  - Protect solutions from light.
  - **Acid stage**
    - **Acid stage medium:** 0.1 N hydrochloric acid; 750 mL
    - **Apparatus 2:** 50 rpm
    - **Time:** 2 h
  - Determine the percentage of the labeled amount of mycophenolic acid (C$_{17}$H$_{29}$O$_{7}$) dissolved by using one of the following procedures.

Spectrophotometric procedure
(See Ultraviolet-Visible Spectroscopy (857).)
- **Standard solution:** Transfer USP Mycophenolate Sodium RS to a suitable volumetric flask, dissolve in methanol equivalent to 5% of the final volume, and dilute with Acid stage medium to volume to obtain the solution having the following concentrations:
  - For Tablets labeled to contain 180 mg of mycophenolic acid: 0.0128 mg/mL of USP Mycophenolate Sodium RS
  - For Tablets labeled to contain 360 mg of mycophenolic acid: 0.0256 mg/mL of USP Mycophenolate Sodium RS
- **Sample solution:** Centrifuge portions of the solution under test or pass through a suitable glass fiber filter of 1-µm pore size.

Instrumental conditions
- **Mode:** UV
- **Analytical wavelength:** 250 nm
- **Cell path length**
  - For Tablets containing 180 mg of mycophenolic acid per Tablet: 0.2 cm
  - For Tablets containing 360 mg of mycophenolic acid per Tablet: 0.1 cm
- **Blank:** Acid stage medium

**Analysis**
- **Samples:** Standard solution and Sample solution
Calculate the percentage of the labeled amount of mycophenolic acid (C_{17}H_{20}O_{3}) dissolved:

\[
\text{Result} = \left(\frac{A_u}{A_s}\right) \times \left(\frac{C_s}{L}\right) \times V \times \left(\frac{M_{r1}}{M_{r2}}\right) \times 100
\]

\( A_u \) = absorbance of the Sample solution
\( A_s \) = absorbance of the Standard solution
\( C_s \) = concentration of USP Mycophenolate Sodium RS in the Standard solution (mg/mL)
\( L \) = label claim (mg/Tablet)
\( V \) = volume of Acid stage medium, 750 mL
\( M_{r1} \) = molecular weight of mycophenolic acid, 320.34
\( M_{r2} \) = molecular weight of mycophenolate sodium, 342.32

Chromatographic procedure

**Solution A:** Solution of 4 mL of triethylamine in 1300 mL of water. Adjust with phosphoric acid to a pH of 5.3.

**Mobile phase:** Acetonitrile and Solution A (35:65)

**Diluent:** Mixture of 0.1 N hydrochloric acid and 0.2 M sodium phosphate (75:25), adjusted with either 2 M sodium hydroxide or hydrochloric acid to a pH of 6.8

**Standard solution:** Solution of USP Mycophenolate Sodium RS in Diluent, equivalent to (L/1000) mg/mL of mycophenolic acid, where \( L \) is the label claim in mg/Tablet

**Sample solution:** Mix 10 mL of the solution under test and 10 mL of 0.2 M sodium phosphate.

**Chromatographic system**

(See Chromatography (621), System Suitability.)

**Mode:** LC

**Detector:** UV 250 nm

**Column:** 4.6-mm × 25-cm; 5-μm packing L11

**Temperatures**

- Autosampler: 4°
- Column: 45°

**Flow rate:** 1.5 mL/min

**Injection volume:** 5 μL

**System suitability**

**Sample:** Standard solution

**Suitability requirements**

- Tailing factor: NMT 2.0
- Relative standard deviation: NMT 2.0%

**Analysis**

**Samples:** Standard solution and Sample solution

Calculate the percentage of the labeled amount of mycophenolic acid (C_{17}H_{20}O_{3}) dissolved:

\[
\left(\frac{r_u}{r_s}\right) \times \left(\frac{C_s}{L}\right) \times V \times \left(\frac{M_{r1}}{M_{r2}}\right) \times 100
\]

\( r_u \) = peak response from the Sample solution
\( r_s \) = peak response from the Standard solution
\( C_s \) = concentration of USP Mycophenolate Sodium RS in the Standard solution (mg/mL)
\( L \) = label claim (mg/Tablet)
\( V \) = volume of Acid stage medium, 750 mL
\( D \) = dilution factor for the Sample solution
\( M_{r1} \) = molecular weight of mycophenolic acid, 320.34
\( M_{r2} \) = molecular weight of mycophenolate sodium, 342.32

**Tolerances:** NMT 5% of the labeled amount of mycophenolic acid (C_{17}H_{20}O_{3}) is dissolved.

**Buffer stage**

**Buffer stage medium:** After 2 h, continue with a pH 6.8 phosphate buffer as follows. Add 250 mL of 0.2 M sodium phosphate and adjust, if necessary, with 2 M sodium hydroxide or hydrochloric acid to a pH of 6.8, 1000 mL. [NOTE—If the volume of the sample withdrawn is greater than 2 mL, replace it with Acid stage medium. Alternatively, increase the volume of 0.2 M sodium phosphate being added to obtain the final volume of 1000 mL and adjust, if necessary, with 2 M sodium hydroxide or hydrochloric acid to a pH of 6.8.]

**Apparatus 2:** 50 rpm

**Time:** 1 h. [NOTE—The total time for this analysis is 3 h, where 2 h is from the Acid stage and 1 h is from the Buffer stage.]

Determine the percentage of the labeled amount of mycophenolic acid (C_{17}H_{20}O_{3}) dissolved by using one of the following procedures.

**Spectrophotometric procedure**

(See Ultraviolet-Visible Spectroscopy (857).)

**Standard solution:** Solution of USP Mycophenolate Sodium RS, equivalent to (L/1000) mg/mL of mycophenolic acid, where \( L \) is the label claim, in mg/Tablet. Prepare the solution as follows. Transfer USP Mycophenolate Sodium RS to a suitable volumetric flask, dissolve in methanol equivalent to 5% of the final volume, and dilute with Buffer stage medium to volume.

**Sample solution:** Centrifuge portions of the solution under test or pass through a suitable glass fiber filter of 1-μm pore size.

**Instrumental conditions**

**Mode:** UV

**Analytical wavelength:** 250 nm

**Cell path length**

- For Tablets containing 180 mg of mycophenolic acid per Tablet: 0.2 cm
- For Tablets containing 360 mg of mycophenolic acid per Tablet: 0.1 cm

**Blank:** Buffer stage medium

**Analysis**

**Samples:** Standard solution and Sample solution

Calculate the percentage of the labeled amount of mycophenolic acid (C_{17}H_{20}O_{3}) dissolved:

\[
\left(\frac{r_u}{r_s}\right) \times \left(\frac{C_s}{L}\right) \times V \times \left(\frac{M_{r1}}{M_{r2}}\right) \times 100
\]

\( r_u \) = absorbance of the Sample solution
\( r_s \) = absorbance of the Standard solution
\( C_s \) = concentration of USP Mycophenolate Sodium RS in the Standard solution (mg/mL)
\( L \) = label claim (mg/Tablet)
\( V \) = volume of Buffer stage medium, 1000 mL
\( M_{r1} \) = molecular weight of mycophenolic acid, 320.34
\( M_{r2} \) = molecular weight of mycophenolate sodium, 342.32

**Chromatographic procedure**

**Solution A,** Mobile phase, Standard solution, and Chromatographic system: Proceed as directed under Chromatographic procedure in Acid stage.

**Sample solution:** Pass the solution under test through a suitable polyethersulfone membrane filter of 0.45-μm pore size and discard a few milliliters of the filtrate.

**Analysis**

**Samples:** Standard solution and Sample solution

Calculate the percentage of the labeled amount of mycophenolic acid (C_{17}H_{20}O_{3}) dissolved:

\[
\left(\frac{r_u}{r_s}\right) \times \left(\frac{C_s}{L}\right) \times V \times \left(\frac{M_{r1}}{M_{r2}}\right) \times 100
\]

\( r_u \) = peak response from the Sample solution
\( r_s \) = peak response from the Standard solution
\( C_s \) = concentration of USP Mycophenolate Sodium RS in the Standard solution (mg/mL)
\( L \) = label claim (mg/Tablet)
Phthalic acid
Phthalic acid monoethyl ester
Mycochromanolate mofetil related compound B
Mycochromanolate
Ethyl ester of mycochromanolate
Any individual unspecified impurity

Tolerances: NLT 80% (Q) of the labeled amount of mycochromanolate acid (C\textsubscript{17}H\textsubscript{16}O\textsubscript{4}) is dissolved.

**Uniformity of Dosage Units (905), Weight Variation:**
Meet the requirements

**Impurities**

- **Organic Impurities:**
  Protect solutions from light.
  Mobile phase, Diluent, Standard solution, and Chromatographic system: Proceed as directed in the Assay.
  System suitability solution: 0.36 \(\mu\)g/mL of USP Mycochromanolate Sodium RS in Diluent. Stir magnetically for at least 60 min to aid dissolution.
  Sensitivity solution: 0.18 \(\mu\)g/mL of USP Mycochromanolate Sodium RS in Diluent from Standard solution
  System suitability
  Samples: Standard solution, System suitability solution, and Sensitivity solution
  Suitability requirements
  Resolution: NLT 1.5 between the mycochromanolate mofetil related compound B and mycochromanolate peaks, System suitability solution
  Tailing factor: NMT 2.0, Standard solution
  Relative standard deviation: NMT 2.0%, Standard solution
  Signal-to-noise ratio: NLT 10, Sensitivity solution

**Analysis**

Samples: Standard solution and Sample solution
Calculate the percentage of each impurity in the portion of Tablets taken:

\[ \text{Result} = \left( \frac{r_s}{r_U} \right) \times \left( \frac{C_U}{C_5} \right) \times \left( \frac{M_{12}}{M_{11}} \right) \times 100 \]

\(r_U\) = peak response of each individual impurity from the Sample solution
\(r_s\) = peak response of mycochromanolate from the Standard solution
\(C_5\) = concentration of USP Mycochromanolate Sodium RS in the Standard solution (mg/mL)
\(C_U\) = nominal concentration of mycochromanolate acid in the Sample solution (mg/mL)
\(M_{11}\) = molecular weight of mycochromanolate acid, 320.34
\(M_{12}\) = molecular weight of mycochromanolate sodium, 342.32

Acceptance criteria: See Table 2. The reporting threshold is 0.05%.

**Additional Requirements**

- **Packaging and Storage:** Preserve in tight containers, and protect from moisture. Store at controlled room temperature.
- **USP Reference Standards (11)**
  USP Mycochromanolate Mofetil Related Compound B RS (RS)-7-Hydroxy-5-methoxy-4-methyl-6-[2-(5-methyl-2-oxo-tetrahydrofuran-5-yi)ethyl]-3H-isobenzofuran-1-one.
  \(\text{C}_{17}\text{H}_{20}\text{O}_6\) 320.34
  USP Mycochromanolate Sodium RS

**Oseltamir Phosphate**

\(\text{C}_{28}\text{H}_{34}\text{N}_2\text{O}_9\cdot\text{H}_2\text{PO}_4\) 410.40
[3R-(3a,4b,5S)]-Ethyl 4-(acetylamino)-5-amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylate phosphate (1:1);
Ethyl (3R,4S,5S)-4-acetamido-5-amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylate, phosphate (1:1) \([204255-11-8]\).

**Definition**

Oseltamir Phosphate contains NLT 98.0% and NMT 101.5% of \(\text{C}_{28}\text{H}_{34}\text{N}_2\text{O}_9\cdot\text{H}_2\text{PO}_4\), calculated on the anhydrous basis.

**Identification**

- **A. Spectroscopic Identification Tests (197), Infrared Spectroscopy:** 197M\(\alpha\) [CN 1-May-2020]
- **B.** The retention time of the major peak of the Sample solution corresponds to that of the Standard solution, as obtained in the Assay.

**Assay**

- **Procedure**
  Solution A: Dissolve 6.8 g of potassium dihydrogen phosphate in 980 mL of water. Adjust with 1 M potassium hydroxide solution to a pH of 6.0, and dilute with water to 1 L.
  Mobile phase: Methanol, acetonitrile, and Solution A (245:135:620)
  Diluent: Methanol, acetonitrile, and water (245:135:620)
  Standard solution: 1 mg/mL of USP Oseltamir Phosphate RS in Diluent
  Sample solution: 1 mg/mL of Oseltamir Phosphate in Diluent

<table>
<thead>
<tr>
<th>Name</th>
<th>Relative Retention Time</th>
<th>Acceptance Criteria, NMT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phthalic acid (a)</td>
<td>0.30</td>
<td>—</td>
</tr>
<tr>
<td>Phthalic acid monoethyl ester (b)</td>
<td>0.55</td>
<td>—</td>
</tr>
<tr>
<td>Mycochromanolate mofetil related compound B</td>
<td>0.90</td>
<td>0.2</td>
</tr>
<tr>
<td>Mycochromanolate</td>
<td>1.0</td>
<td>—</td>
</tr>
<tr>
<td>Ethyl ester of mycochromanolate (c)</td>
<td>2.3</td>
<td>0.2</td>
</tr>
<tr>
<td>Any individual unspecified impurity</td>
<td>—</td>
<td>0.1</td>
</tr>
</tbody>
</table>

**Table 2 (continued)**

<table>
<thead>
<tr>
<th>Name</th>
<th>Relative Retention Time</th>
<th>Acceptance Criteria, NMT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total impurities</td>
<td>—</td>
<td>1.0</td>
</tr>
</tbody>
</table>

\(a\) It is a process impurity and is listed for identification only. It is controlled in the drug substance. It is not reported for the drug product and should not be included in the total impurities.

\(b\) 2-(Ethoxycarbonyl)benzoic acid.

\(c\) Ethyl (6-6-(4-hydroxy-6-methoxy-7-methyl-3-dihydroisobenzofuran-5-yl)-4-methylhex-4-enolate.

Published on March 26, 2020
Chromatographic system  
(See Chromatography (621), System Suitability.)
Mode: LC
Detector: UV 207 nm
Column: 4.6-mm x 25-cm; packing L7
Column temperature: 50°
Flow rate: 1.2 mL/min
Injection size: 15 µL
System suitability
Sample: Standard solution
Suitability requirements
Tailing factor: NMT 2.0
Relative standard deviation: NMT 2.0%
Analysis
Samples: Standard solution and Sample solution
Calculate the percentage of Oseltamivir Phosphate in the portion of Oseltamivir Phosphate taken:

\[ \text{Result} = \left( \frac{r_U}{r_S} \right) \times \left( \frac{C_S}{C_U} \times \left( \frac{1}{F} \right) \times 100 \right) \]

**Acceptance criteria:** 98.0%–101.5% on the anhydrous basis

### IMPURITIES

#### ORGANIC IMPURITIES

- Procedure 1
  - Solution A, Mobile phase, Diluent, Standard solution, Sample solution, and Chromatographic system: Proceed as directed in the Assay.

Analysis

Sample: Sample solution
Calculate the percentage of each impurity in the portion of Oseltamivir Phosphate taken:

\[ \text{Result} = \left( \frac{r_U}{r_S} \right) \times \left( \frac{C_S}{C_U} \times \left( \frac{1}{F} \right) \times 100 \right) \]

**Acceptance criteria**
- Individual impurities: See Impurity Table 1.
- Total impurities: NMT 0.7%

#### Impurity Table 1

<table>
<thead>
<tr>
<th>Name</th>
<th>Relative Retention Time</th>
<th>Relative Response Factor</th>
<th>Acceptance Criteria, NMT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oseltamivir acid</td>
<td>0.17</td>
<td>1.4</td>
<td>0.3</td>
</tr>
<tr>
<td>Oseltamivir phenol</td>
<td>0.51</td>
<td>2.7</td>
<td>0.1</td>
</tr>
<tr>
<td>Oseltamivir phosphate</td>
<td>1.00</td>
<td>1.0</td>
<td>—</td>
</tr>
<tr>
<td>Unspecified impurity</td>
<td>—</td>
<td>1.0</td>
<td>0.1</td>
</tr>
</tbody>
</table>

#### Impurity Table 1 (continued)

<table>
<thead>
<tr>
<th>Name</th>
<th>Relative Retention Time</th>
<th>Relative Response Factor</th>
<th>Acceptance Criteria, NMT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total unspecified impurity</td>
<td>—</td>
<td>—</td>
<td>0.4</td>
</tr>
</tbody>
</table>

a (3R,4R,5S)-4-Acetylamino-5-amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylic acid.
b 4-Acetylamino-3-hydroxybenzoic acid ethyl ester.

- Procedure 2: Oseltamivir Related Compound A
  - Buffer: 1.54 g/L of ammonium acetate in water
  - Mobile phase: Acetonitrile, water, and Buffer (3:6:1)
  - Stock solution A: 50 µg/mL of USP Oseltamivir Related Compound A RS, prepared as follows: Dissolve in alcohol, using 5% of final volume, and dilute with water to volume.

Solution A: 1 µg/mL of USP Oseltamivir Related Compound A RS in water from Stock solution A

Analysis

Sample solution: 10 mg/mL of Oseltamivir Phosphate in water

Chromatographic system  
(See Chromatography (621), System Suitability.)
Mode: LC
Detector: UV 210 nm and mass spectrometer
Column: 3.0-mm x 5-cm; 5-µm packing L1
Flow rate: 1.5 mL/min
Injection size: 1 µL
Temperature: 40°

Use electrospray (+) ionization, a selected ion monitoring mode with m/z of 356.2 (protonated oseltamivir related compound A). Adjust the dwell time, fragmentation voltage, drying gas temperature, drying gas flow, nebulizer pressure, and capillary voltage as appropriate for an optimal response. [Note—A postcolumn flow splitter with a split ratio of about 3:1 is used.]

System suitability
Sample: Standard solution
[Note—The relative retention time for oseltamivir related compound A versus oseltamivir is about 2.6.]

**Suitability requirements**

Resolution: The oseltamivir related compound A peak (detected by MD-SIM mode) and the oseltamivir peak (detected by UV) are baseline resolved. [Note—The resolution of the two components minimizes background noise and ion suppression effects for the trace of oseltamivir related compound A by the oseltamivir matrix.]

**Relative standard deviation:** NMT 15.0%, oseltamivir related compound A peak

Analysis

Samples: Standard solution and Sample solution
Calculate the percentage of oseltamivir related compound A in the portion of Oseltamivir Phosphate taken:

\[ \text{Result} = \left( \frac{r_U}{r_S} \right) \times \left( \frac{C_S}{C_U} \times \left( \frac{1}{F} \right) \times 100 \right) \]

**Acceptance criteria:** NMT 0.01%
• Procedure 3: Limit Of Tributyl Phosphine Oxide

Blank: Transfer 1.0 mL of suitable derivatizing reagent\(^1\) to a vial. Close the vial, shake, and heat for 20 min at 60°. Centrifuge the pyridinium salt precipitate.

**Standard stock solution 1:** 21 mg/mL of USP Tributyl Phosphine Oxide RS in pyridine

**Standard stock solution 2:** 21 mg/mL of USP Oseltamivir Phosphate RS in suitable derivatizing reagent. Close the vial, mix, and heat for 20 min at 60°. Centrifuge the pyridinium salt precipitate.

**Standard solution:** 21 µg/mL each of USP Tributyl Phosphine Oxide RS and USP Oseltamivir Phosphate RS in pyridine from Standard stock solution 1 and Standard stock solution 2, respectively.

**Sample solution:** Transfer 15 mg of Oseltamivir Phosphate to a vial. Add 1.0 mL of suitable derivatizing reagent. Close the vial, mix, and heat for 20 min to 60°. Centrifuge the pyridinium salt precipitate.

**Chromatographic system**

(See Chromatography (621), System Suitability.)

**Mode:** GC

**Detector:** Flame ionization

**Column:** 0.32-mm × 30-m capillary column coated with a 0.25-µm phase G1

**Split ratio:** 1:50

**Split flow:** 64 mL/min

**Injection size:** 1 µL

**Temperature**

Detector: 260°

Injection port: 260°

**Column:** See the temperature program table below.

<table>
<thead>
<tr>
<th>Initial Temperature (°)</th>
<th>Temperature Ramp (°/min)</th>
<th>Final Temperature (°)</th>
<th>Hold Time at Final Temperature (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>180</td>
<td>0</td>
<td>180</td>
<td>2</td>
</tr>
<tr>
<td>180</td>
<td>8</td>
<td>250</td>
<td>10</td>
</tr>
</tbody>
</table>

**Linear velocity:** 27 cm/s

**Carrier gas:** Helium

**System suitability**

**Sample:** Standard solution

[Note—The relative retention times for tributyl phosphine oxide and oseltamivir phosphate are about 0.54 and 1.00, respectively.]

**Suitability requirements**

Relative standard deviation: NMT 10.0% for the tributyl phosphine oxide and oseltamivir phosphate peaks

**Analysis**

**Samples:** Standard solution and Sample solution

Calculate the percentage of tributyl phosphine oxide in the portion of Oseltamivir Phosphate taken:

\[
\text{Result} = \frac{r_U}{r_S} \times \frac{(C_U)}{(C_S)} \times 100
\]

\( r_U \) = peak response of tributyl phosphine oxide from the Sample solution

\( r_S \) = peak response of tributyl phosphine oxide from the Standard solution

\( C_S \) = concentration of tributyl phosphine oxide in the Standard solution (mg/mL)

\( C_U \) = concentration of Oseltamivir Phosphate in the Sample solution (mg/mL)

**Acceptance criteria:** NMT 0.1%

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\(^1\) Tri-Sil Reagent (product number: 48999 0049001) may be obtained from Pierce: www.piercenet.com.

**SPECIFIC TESTS**

• **WATER DETERMINATION, Method I (921):** NMT 0.5%

• **OPTICAL ROTATION, Specific Rotation (781S):** Sample solution: 10 mg/mL in water

**Acceptance criteria:** Between –30.7 and –32.6

**ADDITIONAL REQUIREMENTS**

• **PACKAGING AND STORAGE:** Preserve in well-closed containers. Store at 25°, excursions permitted between 15° and 30°.

• **USP REFERENCE STANDARDS (11):**

  - USP Oseltamivir Phosphate RS
  - USP Oseltamivir Related Compound A RS (35,4R,5S)-Ethyl 4-acetamido-5-amino-2-azido-3-(pentan-3-yloxy)cyclohexanecarboxylate.

  \( \text{C}_13\text{H}_{26}\text{N}_4\text{O}_5 \) 355.43

  USP Tributyl Phosphine Oxide RS

  \( \text{C}_8\text{H}_{32}\text{OP} \) 218.32

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**Oseltamivir Phosphate Capsules**

**DEFINITION**

Oseltamivir Phosphate Capsules contain Oseltamivir Phosphate equivalent to NLT 90.0% and NMT 110.0% of the labeled amount of oseltamivir (\( \text{C}_13\text{H}_{26}\text{N}_4\text{O}_5 \)).

**IDENTIFICATION**

- The retention time of the major peaks of the Sample solution corresponds to those of the Standard solution, as obtained in the Assay.

**ASSAY**

- **Oseltamivir**

  **Solution A:** Dissolve 6.8 g of potassium dihydrogen phosphate in 980 mL of water. Adjust with 1 M potassium hydroxide solution to a pH of 6.0, and dilute with water to 1 L.

  **Mobile phase:** Methanol, acetonitrile, and Solution A (245:135:620)

  **Diluent:** Methanol, acetonitrile, and 0.01 N phosphoric acid (245:135:620)

  **Standard solution:** 1 mg/mL of USP Oseltamivir Phosphate RS in Diluent

  **Sample solution:** Weigh the contents of 20 Capsules, and mix. Prepare the equivalent of about 1 mg of oseltamivir phosphate per mL, based on the label claim, by first dispersing a suitable portion of the powder in about 40% of the flask volume of Diluent using an ultrasonic bath for about 20 min, and diluting with Diluent to volume. Centrifuge an aliquot of this solution, and use the supernatant.

**Chromatographic system**

(See Chromatography (621), System Suitability.)

**Mode:** LC

**Detector:** UV 207 nm

**Column:** 4.6-mm × 25-cm; packing L7

**Column temperature:** 50°

**Flow rate:** 1.2 mL/min

**Injection size:** 15 µL

**System suitability**

**Sample:** Standard solution

**Suitability requirements**

**Tailing factor:** NMT 2.0

**Relative standard deviation:** NMT 2.0%

**Analysis**

**Samples:** Standard solution and Sample solution

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Published on March 26, 2020
Calculate the percentage of the labeled amount of oseltamivir \((C_{17}H_{28}N_{2}O_{5})\) in the portion of Capsules taken:

\[ \text{Result} = \frac{(r_1/r_2) \times (C_d/C_0) \times (M_1/M_2) \times 100}{U \times r_1} \]

- \(r_1\) = peak response from the Sample solution
- \(r_2\) = peak response from the Standard solution
- \(C_d\) = concentration of USP Oseltamivir Phosphate RS in the Standard solution (mg/mL)
- \(C_0\) = nominal concentration of oseltamivir in the Sample solution (mg/mL)
- \(M_1\) = molecular weight of oseltamivir, 312.40
- \(M_2\) = molecular weight of oseltamivir phosphate, 410.40

Acceptance criteria: 90.0%–110.0%

### PERFORMANCE TESTS

#### Dissolution (711)

**Medium:** 0.1 N hydrochloric acid; 900 mL

**Apparatus 2:** 50 rpm

**Time:** 20 min

**Detector:** UV 240 nm

**Standard solution:** Prepare a solution in Medium having a known concentration of about 0.11 mg/mL of USP Oseltamivir Phosphate RS. Quantitatively dilute a portion of this solution with Medium to obtain a solution having a known concentration similar to the expected concentration in the solution under test.

**Sample solution:** Pass a portion of the solution under test through a suitable filter of 1-µm pore size.

**Excipients solution:** Suspend an amount of the placebo mixture equivalent to the weight of the excipients in one dosage unit and one empty Capsule shell in 900 mL of Medium. Heat to 37°, and filter.

**Analysis**

**Samples:** Medium, Standard solution, Sample solution, and Excipients solution

Determine the amount of oseltamivir phosphate \((C_{17}H_{28}N_{2}O_{5} \cdot H_2PO_4)\) dissolved by measuring the absorbance at about 240 nm of the Sample solution and Excipients solution in comparison with the Standard solution, using the Medium as the blank. Calculate the percentage of oseltamivir phosphate dissolved:

\[ \text{Result} = \frac{(A_d - A_0) \times C_1 \times V \times 100} {A_1 \times L} \]

- \(A_d\) = absorbance of the Sample solution
- \(A_0\) = absorbance of the Excipients solution
- \(C_1\) = concentration of USP Oseltamivir Phosphate RS in the Standard solution
- \(V\) = volume of Medium, 900 mL
- \(A_1\) = absorbance of the Standard solution
- \(L\) = label claim for oseltamivir phosphate (mg/Capsule)

**Tolerances:** NLT 75% (Q) of the labeled amount of oseltamivir phosphate is dissolved.

**Uniformity of Dosage Units** (905): Meet the requirements

### IMPURITIES

#### Organic Impurities

**Solution A, Mobile phase, Diluent, Standard solution, Sample solution, Chromatographic system, and System suitability:** Proceed as directed in the Assay.

**Analysis**

Samples: Standard solution and Sample solution

Calculate the percentage of individual impurities in the portion of Capsules taken:

\[ \text{Result} = \frac{(r_1/r_2) \times (C_d/C_0) \times (1/F) \times (M_1/M_2) \times 100}{U \times r_1} \]

- \(r_1\) = peak response from each individual impurity from the Sample solution
- \(r_2\) = peak response from the Standard solution
- \(C_d\) = concentration of USP Oseltamivir Phosphate RS in the Standard solution (mg/mL)
- \(C_0\) = nominal concentration of oseltamivir in the Sample solution (mg/mL)
- \(F\) = relative response factor from Table 1
- \(M_1\) = molecular weight of oseltamivir phosphate, 410.40
- \(M_2\) = molecular weight of oseltamivir phosphate, 312.40

**Acceptance criteria:** See Table 1.

<table>
<thead>
<tr>
<th>Name</th>
<th>Relative Retention Time</th>
<th>Relative Response Factor</th>
<th>Acceptance Criteria, NMT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Impurity A&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.18</td>
<td>1.4</td>
<td>2.0</td>
</tr>
<tr>
<td>Impurity B&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.49</td>
<td>2.7</td>
<td>0.3</td>
</tr>
<tr>
<td>Oseltamivir phosphate</td>
<td>1.00</td>
<td>1.0</td>
<td>—</td>
</tr>
<tr>
<td>Impurity C&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.45</td>
<td>0.9</td>
<td>0.5</td>
</tr>
<tr>
<td>Individual unidentified impurity</td>
<td>—</td>
<td>1.0</td>
<td>0.2</td>
</tr>
<tr>
<td>Total unidentified impurities</td>
<td>—</td>
<td>1.0</td>
<td>0.5</td>
</tr>
<tr>
<td>Total impurities</td>
<td>—</td>
<td>1.0</td>
<td>3.0</td>
</tr>
</tbody>
</table>

<sup>a</sup> (3R,4R,5S)-4-Acetylamino-5-amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylic acid.

<sup>b</sup> 4-Acetylamino-3-hydroxybenzoic acid ethyl ester.

<sup>c</sup> (3R,4R,5S)-4-Amino-5-acetylamino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylic acid ethyl ester.

**ADDITIONAL REQUIREMENTS**

- **Packaging and Storage:** Preserve in well-closed containers. Store at controlled room temperature.

- **USP Reference Standards** (11)

  - USP Oseltamivir Phosphate RS

### Promethazine Hydrochloride

\(C_{17}H_{20}N_2S \cdot HCl\) 320.88

10H-Promethazine-10-ethanamine, \(N,N,N\text{-trimethyl-},\) monohydrochloride, \((\pm)-;\) (\(\pm\))-10-[2-(Dimethylamino)propyl]phenothiazine monohydrochloride \([58-33-3]\).

**Definition**

Promethazine Hydrochloride contains NLT 97.0% and NMT 101.5% of promethazine hydrochloride \((C_{17}H_{20}N_2S \cdot HCl)\), calculated on the dried basis.

**Note**—Throughout the following procedures, protect the samples, the Reference Standards, and the solutions...
containing them, by conducting the procedures without delay under subdued light or using low-actinic glassware.] 

IDENTIFICATION

Change to read:

- **A. SPECTROSCOPIC IDENTIFICATION TESTS (197)**, Infrared Spectroscopy, 197A or 197K (CN 1-May-2020)
- **B. IDENTIFICATION TESTS—GENERAL (191)**, Chloride
- **C.** The retention time of the major peak of the Sample solution corresponds to that of the Standard solution, as obtained in the Assay.

ASSAY

- **PROCEDURE**
  - Diluent: Dissolve 8.2 mL of hydrochloric acid in 1000 mL of water.
  - Mobile phase: Acetonitrile, water, and triethylamine (850:270:1)
  - System suitability stock solution: 1.2 mg/mL of USP Promethazine Related Compound B RS in Diluent. Sonicate to dissolve.
  - Standard solution: 0.1 mg/mL of USP Promethazine Hydrochloride RS in Diluent. Sonicate to dissolve.
  - System suitability solution: 0.09 mg/mL of USP Promethazine Hydrochloride RS and 0.12 mg/mL of USP Promethazine Related Compound B RS in Diluent from the Standard solution and System suitability stock solution, respectively.
  - Sample solution: 0.1 mg/mL of Promethazine Hydrochloride in Diluent. Sonicate to dissolve.

Chromatographic system

(See Chromatography (621), System Suitability.)

Mode: LC
Detector: UV 254 nm
Column: 3.9-mm × 30-cm; 10-µm packing L1
Flow rate: 2.5 mL/min
Injection volume: 20 µL
Run time: NLT 2.5 times the retention time of promethazine

System suitability

Samples: System suitability solution and Standard solution
[NOTE—The relative retention times for promethazine related compound B and promethazine are 0.82 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 1.5 between promethazine and promethazine related compound B, System suitability solution
Tailing factor: NMT 1.5, Standard solution
Relative standard deviation: NMT 2.0%, Standard solution

Analysis

Samples: Standard solution and Sample solution
Calculate the percentage of promethazine hydrochloride (C_{18}H_{22}N_{2}S·HCl) in the portion of Promethazine Hydrochloride taken:

\[
\text{Result} = \left( \frac{r_0}{r_s} \right) \times \left( \frac{C_t}{C_u} \right) \times 100
\]

\[
r_0 = \text{peak response from the Sample solution}
\]

\[
r_s = \text{peak response from the Standard solution}
\]

\[
C_t = \text{concentration of USP Promethazine Hydrochloride RS in the Standard solution (mg/mL)}
\]

\[
C_u = \text{concentration of Promethazine Hydrochloride in the Sample solution (mg/mL)}
\]

Acceptance criteria: 97.0%–101.5% on the dried basis

IMPURITIES

- **RESIDUE ON IGNITION** (281): NMT 0.1%
- **ORGANIC IMPURITIES**
  - Diluent: Methanol and triethylamine (999:1)
  - Buffer: 3.7 g/L of ammonium acetate in water
  - Solution A: Acetonitrile and Buffer (300:700)
  - Solution B: Acetonitrile
  - Mobile phase: See Table 1.

<table>
<thead>
<tr>
<th>Table 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time (min)</td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td>10</td>
</tr>
<tr>
<td>18</td>
</tr>
<tr>
<td>18.1</td>
</tr>
<tr>
<td>25</td>
</tr>
</tbody>
</table>

System suitability stock solution: 0.5 mg/mL of USP Promethazine Related Compound B RS in Diluent
Standard stock solution: 0.5 mg/mL of USP Promethazine Hydrochloride RS in Diluent
System suitability solution: 5 µg/mL each of USP Promethazine Hydrochloride RS and USP Promethazine Related Compound B RS from the Standard stock solution and System suitability stock solution, respectively
Standard solution: 5 µg/mL of USP Promethazine Hydrochloride RS from the Standard stock solution
Sensitivity solution: 0.25 µg/mL of USP Promethazine Hydrochloride RS from the Standard stock solution
Sample solution: 0.5 mg/mL of Promethazine Hydrochloride in Diluent

Chromatographic system

(See Chromatography (621), System Suitability.)

Mode: LC
Detector: UV 234 and 249 nm
Column: 4.6-mm × 15-cm; 5-µm packing L1
Column temperature: 30°C
Flow rate: 1.4 mL/min
Injection volume: 15 µL

System suitability

Samples: System suitability solution, Standard solution, and Sensitivity solution
[NOTE—See Table 2 for the relative retention times.]

Suitability requirements

Resolution: NLT 5.0 between promethazine and promethazine related compound B, System suitability solution
Relative standard deviation: NMT 3.0% at 234 and 249 nm, Standard solution
Signal-to-noise ratio: NLT 10 at 234 and 249 nm, Sensitivity solution

Analysis

Samples: Standard solution and Sample solution
Calculate the percentage of promethazine sulfoxide in the portion of Promethazine Hydrochloride taken:

\[
\text{Result} = \left( \frac{r_0}{r_s} \right) \times \left( \frac{C_t}{C_u} \right) \times (1/F) \times 100
\]

\[
r_0 = \text{peak response of promethazine sulfoxide at 234 nm from the Sample solution}
\]

\[
r_s = \text{peak response of promethazine hydrochloride at 234 nm from the Standard solution}
\]

\[
C_t = \text{concentration of USP Promethazine Hydrochloride RS in the Standard solution (mg/mL)}
\]

\[
C_u = \text{concentration of Promethazine Hydrochloride in the Sample solution (mg/mL)}
\]
Promethazine Hydrochloride Tablets

**DEFINITION**
Promethazine Hydrochloride Tablets contain NLT 95.0% and NMT 110.0% of the labeled amount of promethazine hydrochloride (C₁₀H₁₇N₂S · HCl).

[Notes—Throughout the following procedures, protect the samples, the Reference Standards, and the solutions containing them, by conducting the procedures without delay under subdued light or using low-actinic glassware.]

**IDENTIFICATION**

- **A.**
  - **Standard solution:** In a separator, dissolve 50 mg of USP Promethazine Hydrochloride RS in 40 mL of dilute hydrochloric acid (1 in 1000).
  - **Sample solution:** Shake a quantity of powdered Tablets, equivalent to 50 mg of promethazine hydrochloride, with 30 mL of chloroform, and filter into a beaker. Evaporate the chloroform, dissolve the residue in 40 mL of dilute hydrochloric acid (1 in 1000), and transfer the liquid to a separator.

- **Analysis:** Separately treat the **Sample solution** and the **Standard solution** as follows. Add 2 mL of 1 N sodium hydroxide and 15 mL of carbon disulfide to the separators, and shake for 2 min. Centrifuge if necessary to clarify the lower phase, and pass through a dry filter, collecting the filtrate from each separator in a small flask provided with a glass stopper. Reduce the volume of the carbon disulfide extracts to 4–5 mL, and proceed as directed in **Identification**—Organic Nitrogenous Bases (181), beginning with “Determine the absorption spectra”.

  - **Acceptance criteria:** Meet the requirements

- **B.**
  - The retention time of the major peak of the **Sample solution** corresponds to that of the **Standard solution**, as obtained in the **Assay**.

**ASSAY**

- **PROCEDURE**
  - **Diluent:** Dissolve 8.2 mL of hydrochloric acid in 1000 mL of water.
  - **Mobile phase:** Acetonitrile, water, and triethylamine (850:270:1)
  - **System suitability stock solution:** 1.2 mg/mL of USP Promethazine Related Compound B RS in Diluent. Sonicate to dissolve.
  - **Standard solution:** 0.1 mg/mL of USP Promethazine Hydrochloride RS in Diluent. Sonicate to dissolve.
  - **System suitability solution:** 0.09 mg/mL of USP Promethazine Hydrochloride RS and 0.12 mg/mL of USP Promethazine Related Compound B RS in Diluent from the **System suitability stock solution**, respectively
  - **Sample stock solution:** Nominally 2.5–5.0 mg/mL of promethazine hydrochloride prepared as follows. Transfer 20 Tablets to a volumetric flask of an appropriate size and add 50% of the flask volume of Diluent. Sonicate with swirling for NLT 20 min, or until the Tablets have fully disintegrated. Shake the flask for NLT 15 min and dilute with Diluent to volume.

- **Sample solution:** Nominally 0.1 mg/mL of promethazine hydrochloride in Diluent from the **Sample stock solution**. Pass a portion through a filter of 0.45-µm pore size and use the clear filtrate.

  - **Chromatographic system**
    (See Chromatography (621), System Suitability.)
    - **Mode:** LC
    - **Detector:** UV 254 nm
    - **Column:** 3.9-mm × 30-cm; 10-µm packing L1
    - **Flow rate:** 2.5 mL/min

---

**Table 2**

<table>
<thead>
<tr>
<th>Name</th>
<th>Relative Retention Time</th>
<th>Relative Response Factor</th>
<th>Acceptance Criteria, NMT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Promethazine sulfoxide⁵</td>
<td>0.28</td>
<td>2.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Desmethyl promethazine⁶</td>
<td>0.71</td>
<td>1.0</td>
<td>0.2</td>
</tr>
<tr>
<td>Promethazine</td>
<td>1.0</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Promethazine related compound B</td>
<td>1.3</td>
<td>1.0</td>
<td>0.8</td>
</tr>
<tr>
<td>Phenothiazine</td>
<td>1.7</td>
<td>2.0</td>
<td>0.1</td>
</tr>
<tr>
<td>Any individual unspecified impurity</td>
<td>—</td>
<td>1.0</td>
<td>0.10</td>
</tr>
<tr>
<td>Total impurities</td>
<td>—</td>
<td>—</td>
<td>1.2</td>
</tr>
</tbody>
</table>

⁵ N,N-Dimethyl-1-(10H-phenothiazin-10-yl)propan-2-amine sulfoxide.
⁶ N-Methyl-1-(10H-phenothiazin-10-yl)propan-2-amine.

---

**SPECIFIC TESTS**

- **PH (791)**
  - **Sample solution:** 50 mg/mL of Promethazine Hydrochloride
  - **Acceptance criteria:** 4.0–5.0

- **LOSS ON DRYING (731)**
  - **Analysis:** Dry at 105° for 4 h.
  - **Acceptance criteria:** NMT 0.5%

---

**ADDITIONAL REQUIREMENTS**

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
- **USP REFERENCE STANDARDS (11)**
  - USP Promethazine Hydrochloride RS
  - USP Promethazine Related Compound B RS
  - Isopromethazine hydrochloride; N,N-Dimethyl-2-(10H-phenothiazin-10-yl)propan-1-amine hydrochloride; C₁₀H₁₇N₂S · HCl 320.88

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Published on March 26, 2020
**Injection volume:** 20 µL  
**Run time:** NLT 2.5 times the retention time of promethazine  
**System suitability**  
**Samples:** System suitability solution and Standard solution  

[NOTE—The relative retention times for promethazine related compound B and promethazine are 0.82 and 1.0, respectively.]

**Suitability requirements**  
**Resolution:** NLT 1.5 between promethazine and promethazine related compound B, System suitability solution  
**Tailing factor:** NMT 1.5, Standard solution  
**Relative standard deviation:** NMT 2.0%, Standard solution  

**Analysis**  
**Samples:** Standard solution and Sample solution  

Calculate the percentage of the labeled amount of promethazine hydrochloride \((C_{17}H_{20}N_2S \cdot HCl)\) in the portion of Tablets taken:

\[
\text{Result} = \left( \frac{r_u}{r_s} \right) \times \left( \frac{C_s}{C_u} \right) \times 100
\]

- \(r_u\) = peak response from the Sample solution  
- \(r_s\) = peak response from the Standard solution  
- \(C_s\) = concentration of USP Promethazine Hydrochloride RS in the Standard solution (mg/mL)  
- \(C_u\) = nominal concentration of promethazine hydrochloride in the Sample solution (mg/mL)  

**Acceptance criteria:** 95.0%–110.0%

**PERFORMANCE TESTS**  
**Dissolution (711)**

**Test 1**  
**Medium:** 0.01 N hydrochloric acid; 900 mL  
**Apparatus 1:** 100 rpm  
**Time:** 45 min  
**Standard solution:** Prepare a solution with a known concentration of USP Promethazine Hydrochloride RS in Medium.  
**Sample solution:** A filtered portion of the solution under test, suitably diluted with Medium  
**Instrumental conditions**  
**Mode:** UV  
**Analytical wavelength:** Absorption maximum at about 249 nm  
**Analysis**  
**Samples:** Standard solution and Sample solution  

Calculate the percentage of the labeled amount of promethazine hydrochloride \((C_{17}H_{20}N_2S \cdot HCl)\) dissolved:

\[
\text{Result} = \left( \frac{A_u}{A_s} \right) \times \frac{S}{C_s} \times V \times \left(1/L\right) \times 100
\]

- \(A_u\) = absorbance of the Sample solution  
- \(A_s\) = absorbance of the Standard solution  
- \(C_s\) = concentration of USP Promethazine Hydrochloride RS in the Standard solution (mg/mL)  
- \(V\) = volume of Medium, 900 mL  
- \(L\) = label claim (mg/Tablet)  

**Tolerances:** NLT 80% (Q) of the labeled amount of promethazine hydrochloride \((C_{17}H_{20}N_2S \cdot HCl)\) is dissolved.  

**Uniformity of Dosage Units (905):** Meet the requirements

**Impurities**

**Organic impurities**  
**Diluent:** Methanol and triethylamine (999:1)  
**Buffer:** 3.7 g/L of ammonium acetate in water  
**Solution A:** Buffer and acetonitrile (700:300)  
**Solution B:** Acetonitrile  
**Mobile phase:** See Table 1.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Solution A (%)</th>
<th>Solution B (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>60</td>
<td>40</td>
</tr>
<tr>
<td>18</td>
<td>60</td>
<td>40</td>
</tr>
<tr>
<td>18.1</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>25</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

**System suitability stock solution:** 0.5 mg/mL of USP Promethazine Related Compound B RS in Diluent  
**Standard stock solution:** 0.5 mg/mL of USP Promethazine Hydrochloride RS in Diluent  
**System suitability solution:** 5 µg/mL each of USP Promethazine Hydrochloride RS and USP Promethazine Related Compound B RS from the Standard stock solution and System suitability stock solution, respectively

**Standard solution:** 5 µg/mL of USP Promethazine Hydrochloride RS from the Standard stock solution  
**Sensitivity solution:** 0.25 µg/mL of USP Promethazine Hydrochloride RS from the Standard stock solution
Sample solution: Nominally 0.5 mg/mL of promethazine hydrochloride from powdered Tablets (NLT 20) prepared as follows. Transfer a quantity of powdered Tablets, equivalent to 50 mg of promethazine hydrochloride, to a volumetric flask of appropriate size and add 75% of the flask volume of Diluent. Shake the flask for NLT 5 min and dilute with Diluent to volume. Pass a portion through a suitable filter.

Chromatographic system
(See Chromatography (621), System Suitability.)
Mode: LC
Detector: UV 234 and 249 nm
Column: 4.6-mm × 15-cm; 5-µm packing L1
Column temperature: 30°C
Flow rate: 1.4 mL/min
Injection volume: 15 µL

System suitability
Samples: System suitability solution, Standard solution, and Sensitivity solution
[Note—See Table 2 for the relative retention times.]

Suitability requirements
Resolution: NLT 5.0 between promethazine and promethazine related compound B, System suitability solution
Relative standard deviation: NMT 3.0% at 234 and 249 nm, Standard solution
Signal-to-noise ratio: NLT 10 at 234 and 249 nm, Sensitivity solution

Analysis
Samples: Standard solution and Sample solution
Calculate the percentage of promethazine sulfoxide in the portion of Tablets taken:
\[
\text{Result} = \left(\frac{r_n}{r_s}\right) \times \left(\frac{C_s}{C_n}\right) \times \left(\frac{1}{F}\right) \times 100
\]

Calculate the percentage of all other degradation products in the portion of Tablets taken:
\[
\text{Result} = \left(\frac{r_n}{r_s}\right) \times \left(\frac{C_s}{C_n}\right) \times \left(\frac{1}{F}\right) \times 100
\]

Acceptance criteria: See Table 2. Disregard peaks that are less than 0.05%.

### Table 2

<table>
<thead>
<tr>
<th>Name</th>
<th>Relative Retention Time</th>
<th>Relative Response Factor</th>
<th>Acceptance Criteria, NMT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Promethazine sulfoxide</td>
<td>0.28</td>
<td>2.1</td>
<td>0.5</td>
</tr>
<tr>
<td>Desmethyl promethazine</td>
<td>0.71</td>
<td>1.0</td>
<td>0.5</td>
</tr>
<tr>
<td>Promethazine</td>
<td>1.0</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Promethazine related compound</td>
<td>1.3</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Phenothenzine</td>
<td>1.7</td>
<td>2.0</td>
<td>0.5</td>
</tr>
<tr>
<td>Any individual unspecified degradation product</td>
<td>—</td>
<td>1.0</td>
<td>0.2</td>
</tr>
<tr>
<td>Total degradation products</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

Additional requirements
- **Packaging and Storage:** Preserve in tight, light-resistant containers. Store at controlled room temperature.
- **Labeling:** When more than one Dissolution test is given, the labeling states the test used only if Test 1 is not used.
- **USP Reference Standards (11)**
  - USP Promethazine Hydrochloride RS
  - USP Promethazine Related Compound B RS
  - USP Isopromethazine hydrochloride; N,N-Dimethyl-1-(10H-phenothiazin-10-yl)propan-2-amine hydrochloride;
  - C_{16}H_{24}N_6S · HCl 320.88

**Promethazine Hydrochloride Injection**

**Definition**
Promethazine Hydrochloride Injection is a sterile solution of Promethazine Hydrochloride in Water for Injection. It contains NLT 95.0% and NMT 110.0% of the labeled amount of promethazine hydrochloride (C_{16}H_{24}N_6S · HCl).

[Note—Throughout the following procedures, protect the samples, the Reference Standards, and the solutions containing them, by conducting the procedures without delay under subdued light or using low-actinic glassware.]

**Identification**
- A. The retention time of the major peak of the Sample solution corresponds to that of the Standard solution, as obtained in the Assay.
- B. The UV spectrum of the major peak of the Sample solution corresponds to that of the Standard solution, as obtained in the Assay.

**Assay**
- **Procedure**
  - Buffer: 3.7 g/L of ammonium acetate in water
  - Solution A: Acetonitrile and Buffer (30:70)
  - Mobile phase: See Table 1.
Chromatographic system:

Mobile phase, Diluent, and Chromatographic system:

Proceed as directed in the Assay.

Table 1

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Solution A (%)</th>
<th>Acetonitrile (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>60</td>
<td>40</td>
</tr>
<tr>
<td>18</td>
<td>60</td>
<td>40</td>
</tr>
<tr>
<td>18.1</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>25</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

Diluent: 0.1% triethylamine in methanol

System suitability solution: 1.0 µg/mL each of USP Promethazine Hydrochloride RS and USP Promethazine Related Compound B RS in Diluent

Standard solution: 0.05 mg/mL of USP Promethazine Hydrochloride RS in Diluent

Sample solution: Nominally 0.05 mg/mL of promethazine hydrochloride from a volume of Injection in Diluent

[Note—Sonication may be used in the preparation of System suitability solution, Standard solution, and Sample solution.]

Chromatographic system (See Chromatography (621), System Suitability.)

Mode: LC

Detector: UV 254 nm. For Identification B, use a diode array detector in the range of 200–400 nm.

Column: 4.6-mm × 15-cm; 5-µm packing L1

Temperatures

Column: 30°C

Autosampler: 4°C

Flow rate: 1.4 mL/min

Injection volume: 15 µL

System suitability

Samples: System suitability solution and Standard solution [Note—The relative retention times for promethazine and promethazine related compound B are 1.0 and 1.3, respectively.]

Suitability requirements

Resolution: NLT 5.0 between the promethazine and promethazine related compound B peaks

Relative standard deviation: NMT 2.0% for promethazine peaks

Analysis

Samples: Standard solution and Sample solution

Calculate the percentage of each degradation product in the portion of Injection taken:

Result = \( \left( \frac{r_u}{r_s} \right) \times \left( \frac{C_s}{C_u} \right) \times (1/F) \times 100 \)

Acceptance criteria: See Table 2. Disregard peaks less than 0.05%.

Table 2

<table>
<thead>
<tr>
<th>Name</th>
<th>Relative Retention Time</th>
<th>Relative Response Factor</th>
<th>Acceptance Criteria, NMT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Promethazine sulfoxide(^a)</td>
<td>0.3</td>
<td>0.29</td>
<td>2.6</td>
</tr>
<tr>
<td>Desmethyl promethazine(^b)</td>
<td>0.7</td>
<td>1.1</td>
<td>0.2</td>
</tr>
<tr>
<td>Promethazine</td>
<td>1.0</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Promethazine related com-</td>
<td>1.3</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>pound B(^d)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenothiazine(^c)</td>
<td>1.7</td>
<td>2.3</td>
<td>0.2</td>
</tr>
<tr>
<td>Any individual unspecified degradation product</td>
<td>—</td>
<td>1.0</td>
<td>0.1</td>
</tr>
<tr>
<td>Total degradation products</td>
<td>—</td>
<td>—</td>
<td>2.8</td>
</tr>
</tbody>
</table>

\(^a\) N,N-Dimethyl-1-(10H-phenothiazin-10-yl)propan-2-amine sulfoxide.  
\(^b\) N-Methyl-1-(10H-phenothiazin-10-yl)propan-2-amine.  
\(^c\) This is a process impurity that is controlled in the drug substance and is included for identification only.  
\(^d\) 10H-Phenothiazine.

Specific tests

**Bacterial Endotoxins Test (85):** NMT 5.0 USP Endotoxin Units/mg of promethazine hydrochloride

**pH (791):** 4.0–5.5

**Other Requirements:** It meets the requirements in Injections and Implanted Drug Products (1).
Promethazine Hydrochloride

Suppositories

Promethazine Hydrochloride Suppositories contain not less than 95.0 percent and not more than 110.0 percent of the labeled amount of \( C_{17}H_{20}N_2 S \cdot HCl \).

Packaging and storage—Preserve in tight, light-resistant containers, and store in a cold place.

USP Reference standards (11)—

USP Promethazine Hydrochloride RS

USP Promethazine Related Compound B RS

Isopromethazine hydrochloride;

\( N,N\text{-Dimethyl-}2-(10\text{H}-\text{phenothiazin-10-yl})\text{propan-1-amine hydrochloride} \).

\( C_{17}H_{20}N_2 S \cdot HCl \) 320.88

Additiona1 requirements

- Packaging and Storage: Preserve in single-dose or in multiple-dose containers, preferably of Type I glass, protected from light.
- USP Reference Standards (11)
  - USP Promethazine Hydrochloride RS
  - USP Promethazine Related Compound B RS
  - Isopromethazine hydrochloride;
  - \( N,N\text{-Dimethyl-}2-(10\text{H}-\text{phenothiazin-10-yl})\text{propan-1-amine hydrochloride} \).

- Promethazine Hydrochloride Oral Solution

**Definiton**

Promethazine Hydrochloride Oral Solution contains NLT 90.0% and NMT 110.0% of the labeled amount of promethazine hydrochloride (\( C_{17}H_{20}N_2 S \cdot HCl \)).

**Identification**

- A. The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*.
- B. The UV spectrum of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*.

**Assay**

- Procedure
  - Buffer: 3.7 g/L of ammonium acetate in water
  - Solution A: Acetonitrile and *Buffer* (30:70)
  - Solution B: Acetonitrile
  - Mobile phase: See Table 1.

**Table 1**

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Solution A (%)</th>
<th>Solution B (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>60</td>
<td>40</td>
</tr>
<tr>
<td>18</td>
<td>60</td>
<td>40</td>
</tr>
<tr>
<td>18.1</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>25</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

Diluent: 0.1% triethylamine in methanol

System suitability solution: 1.0 \( \mu \text{g/mL} \) each of USP Promethazine Hydrochloride RS and USP Promethazine Related Compound B RS in *Diluent*

**Standard solution:** 0.05 \( \mu \text{g/mL} \) of USP Promethazine Hydrochloride RS in *Diluent*
Sample solution: Nominally 0.05 mg/mL of promethazine hydrochloride from a volume of Oral Solution in Diluent. Centrifuge for 10 min and use the supernatant.

Chromatographic system
(See Chromatography (621), System Suitability.)
Mode: LC
Detector: UV 254 nm. For Identification test B, use a diode array detector in the range of 200–400 nm.
Column: 4.6-mm × 15-cm; 5-µm packing L1
Temperatures
Autosampler: 4°
Column: 30°
Flow rate: 1.4 mL/min
Injection volume: 15 µL
System suitability
Samples: System suitability solution and Standard solution
Suitability requirements
Resolution: NLT 5.0 between promethazine and promethazine related compound B peaks, System suitability solution
Tailing factor: NMT 2.0, Standard solution
Relative standard deviation: NMT 0.5 between promethazine and promethazine related compound B peaks.
Acceptance criteria:
Chromatographic system:
Sample solution: Nominally 0.05 mg/mL of promethazine hydrochloride from a volume of Oral Solution in Diluent. Centrifuge for 10 min and use the supernatant.

Analysis
Suitability requirements
Resolution: NLT 5.0 between promethazine and promethazine related compound B peaks
Relative standard deviation: NMT 5.0% for promethazine

Acceptance criteria: See Table 2. Disregard peaks less than 0.05%.

Table 2

<table>
<thead>
<tr>
<th>Name</th>
<th>Relative Retention Time</th>
<th>Relative Response Factor</th>
<th>Acceptance Criteria, NMT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Promethazine sulfoxidea</td>
<td>0.3</td>
<td>0.26</td>
<td>1.0</td>
</tr>
<tr>
<td>Desmethyl promethazineb</td>
<td>0.6</td>
<td>1.0</td>
<td>0.2</td>
</tr>
<tr>
<td>Promethazine</td>
<td>1.0</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Promethazine related compound Bc</td>
<td>1.3</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Phenothiazined</td>
<td>1.5</td>
<td>2.2</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Any individual unspecified degradation product — 1.0 0.2

a N,N-Dimethyl-1-(10H-phenothiazin-10-yl)propan-2-amine sulfoxide.
b N-Methyl-1-(10H-phenothiazin-10-yl)propan-2-amine.
c This is a process impurity which is controlled in the drug substance and is included in the table for identification only.
d 10H-Phenothiazine.

ADDITIONAL REQUIREMENTS

• PACKAGING AND STORAGE: Preserve in tight, light-resistant containers.

• USP REFERENCE STANDARDS (11)
  USP Promethazine Hydrochloride RS
  USP Promethazine Related Compound B RS
  Isopromethazine hydrochloride;
  N,N-Dimethyl-2-(10H-phenothiazin-10-yl)propan-1-amine hydrochloride;
  C17H20N5S·HCl 320.88

Ribavirin

\[\text{C}_{13}\text{H}_{17}\text{N}_{2}\text{O}_{4}\] 244.20

1H-1,2,4-Triazole-3-carboxamide, 1-β-D-ribofuranosyl-1H-1,2,4-triazole-3-carboxamide [36791-04-5].
DEFINITION
Ribavirin contains NLT 98.9% and NMT 101.5% of ribavirin (C₉H₁₂N₂O₄), calculated on the dried basis.

IDENTIFICATION
Change to read:

• A. *SPECTROSCOPIC IDENTIFICATION TESTS* (197), Infrared Spectroscopy: 192K (CN 1-May-2020)

• B. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST (201)

Sample solution: 10 mg/mL
Developing solvent system: Acetonitrile and 0.1 M ammonium chloride (9:2)
Spray reagent: Anisaldehyde, sulfuric acid, glacial acetic acid, and alcohol (5:5:1:90)
Analysis: Proceed as directed in the chapter. Allow the plate to air-dry for 15 min, spray with Spray reagent, heat the plate at 110° for 30 min, and locate the spots on the plate by examining the plate in daylight.
Acceptance criteria: Meets the requirements

ASSAY
• PROCEDURE
Mobile phase: Adjust water with sulfuric acid to a pH of 2.5 ± 0.1. Pass through a suitable filter of 0.5-µm or finer pore size.
Standard solution: 0.025 mg/mL of USP Ribavirin RS in Acetonitrile and 0.1 M ammonium chloride (9:2)
Sample solution: 0.025 mg/mL of Ribavirin Tablets in Mobile phase

Chromatographic system
(See Chromatography (621), System Suitability.)
Mode: LC
Detector: UV 207 nm
Column: 7.8-mm × 10-cm; packing L17
Column temperature: 37 ± 0.5°
Flow rate: 1 mL/min
Injection volume: 10 µL
System suitability
Sample: Standard solution
Suitability requirements
Tailing factor: 0.7–1.5
Relative standard deviation: NMT 0.5%
Analysis
Samples: Standard solution and Sample solution
Calculate the percentage of ribavirin (C₉H₁₂N₂O₄) in the portion of Ribavirin taken:

\[
\text{Result} = \left( \frac{r_u}{r_f} \right) \times \left( \frac{C_u}{C_f} \right) \times 100
\]

\[r_u = \text{peak response from the Sample solution}\]
\[r_f = \text{peak response from the Standard solution}\]
\[C_u = \text{concentration of USP Ribavirin RS in the Standard solution (mg/mL)}\]
\[C_f = \text{concentration of Ribavirin in the Sample solution (mg/mL)}\]

Acceptance criteria: 98.9%–101.5% on the dried basis

IMPURITIES
• RESIDUE ON IGNITION (281): NMT 0.25%
• ORGANIC IMPURITIES
Mobile phase, Standard solution, Chromatographic system, and System suitability: Proceed as directed in the ASSAY.
Sample stock solution: 0.5 mg/mL of Ribavirin in Mobile phase
Analysis Sample: Sample stock solution

Calculate the percentage of each peak, other than the solvent peak and the ribavirin peak, in the portion of Ribavirin taken:

\[
\text{Result} = \left( \frac{r_u}{r_f} \right) \times 100
\]

\[r_u = \text{response of the individual peak}\]
\[r_f = \text{sum of the responses of all the peaks}\]

Acceptance criteria
Any individual impurity: NMT 0.25%
Total impurities: NMT 1.0%

SPECIFIC TESTS
• OPTICAL ROTATION, Specific Rotation (781S)
Sample solution: 10 mg/mL
Acceptance criteria: −33.5° to −37.0° (t = 20°)

• pH (791)
Sample solution: 20 mg/mL
Analysis: Add 0.2 mL of a saturated potassium chloride solution to 50 mL of Sample solution.
Acceptance criteria: 4.0–6.5

• LOSS ON DRYING (731)
Analysis: Dry a sample at 105° for 5 h.
Acceptance criteria: NMT 0.5%

ADDITIONAL REQUIREMENTS
• PACKAGING AND STORAGE: Preserve in tight containers.
• USP REFERENCE STANDARDS (11) USP Ribavirin RS

Ribavirin Tablets

DEFINITION
Ribavirin Tablets contain NLT 90.0% and NMT 110.0% of the labeled amount of ribavirin (C₉H₁₂N₂O₄).

IDENTIFICATION
• A. The retention time of the ribavirin peak from the Sample stock solution corresponds to that of the Standard solution, as obtained in the ASSAY.

ASSAY
• PROCEDURE
Buffer: 4.0 g/L of sodium dihydrogen phosphate dihydrate in water. Adjust with 5% sodium hydroxide solution to a pH of 5.0 ± 0.05. Pass the solution through a suitable filter of 0.45-µm pore size.
Mobile phase: Acetonitrile and Buffer (1:49)
Diluent: Acetonitrile and water (3:7)
Standard stock solution: 0.6 mg/mL of USP Ribavirin RS in Diluent
Standard solution: 0.03 mg/mL of USP Ribavirin RS in Mobile phase from the Standard stock solution
Sample stock solution: Transfer a portion of ribavirin, equivalent to 1000 mg of ribavirin from finely powdered Tablets (NLT 10), to a 1000-mL volumetric flask. Add about 750 mL of Diluent, and sonicate with occasional shaking for 30 min. Cool to room temperature, dilute with Diluent to volume, and mix. Centrifuge, and decant the supernatant.
Sample solution: 0.03 mg/mL of ribavirin in Mobile phase from the Sample stock solution. Pass the solution through a suitable filter of 0.45-µm pore size.

Chromatographic system
(See Chromatography (621), System Suitability.)
Mode: LC
Detector: UV 207 nm
Column: 4.6-mm × 25-cm; 5-µm packing L1
Flow rate: 1 mL/min
Injection size: 20 µL
Run time: 10 min

System suitability
Sample: Standard solution

Suitability requirements
Column efficiency: NLT 2000 theoretical plates
Tailing factor: NMT 2.0
Relative standard deviation: NMT 2.0%

Analysis
Samples: Standard solution and Sample solution

Calculate the percentage of ribavirin (C₄H₆N₄O₅) in the portion of Tablets taken:

\[
\text{Result} = \left( \frac{r_u}{r_S} \right) \times \left( \frac{C_S}{C_U} \right) \times 100
\]

- \( r_u \) = peak response of ribavirin from the Sample solution
- \( r_S \) = peak response of ribavirin from the Standard solution
- \( C_S \) = concentration of USP Ribavirin RS in the Standard solution (mg/mL)
- \( C_U \) = nominal concentration of ribavirin in the Sample solution (mg/mL)

Acceptance criteria: 90.0%–110.0%

**PERFORMANCE TESTS**

- **Dissolution (711)**
  - Medium: Water; 900 mL
  - Apparatus 2: 50 rpm
  - Time: 30 min
  - Buffer and Mobile phase: Proceed as directed in the Assay.

Standard solution: 0.22 mg/mL of USP Ribavirin RS in Solution A

Sample solution: Pass the solution through a suitable filter of 0.45-µm pore size.

Chromatographic system
(See Chromatography (621), System Suitability.)

Mode: LC

- Detector: UV 225 nm
- Column: 4.6-mm × 25-cm; 5-µm packing L1
- Flow rate: 1 mL/min
- Injection size: 10 µL

System suitability
Sample: Standard solution

Suitability requirements
Column efficiency: NLT 2000 theoretical plates
Tailing factor: NMT 2.0
Relative standard deviation: NMT 2.0%

Analysis
Calculate the percentage of ribavirin (C₄H₆N₄O₅) dissolved:

\[
\text{Result} = \left( \frac{r_u}{r_S} \right) \times \left( \frac{C_S}{L} \right) \times V \times 100
\]

- \( r_u \) = peak response of ribavirin from the Sample solution
- \( r_S \) = peak response of ribavirin from the Standard solution
- \( C_S \) = concentration of USP Ribavirin RS in the Standard solution (mg/mL)
- \( L \) = label claim (mg/Tablet)
- \( V \) = volume of Medium, 900 mL

Tolerances: NLT 80% (Q) of the labeled amount of ribavirin (C₄H₆N₄O₅) is dissolved.

**Uniformity of Dosage Units** (905): Meet the requirements

### IMPURITIES

**Organic Impurities: Procedure 1**

If uracil and/or uridine are known impurities, Procedure 2 is recommended.

**Solution A**: 3.4 g/L of potassium dihydrogen phosphate in water. Adjust with 5% potassium hydroxide solution to a pH of 5.00 ± 0.05. Pass the solution through a suitable filter of 0.45-µm pore size.

**Solution B**: Acetonitrile

Mobile phase: See **Table 1**.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Solution A (%)</th>
<th>Solution B (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>30</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>40</td>
<td>75</td>
<td>25</td>
</tr>
<tr>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>55</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>56</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>70</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

**Standard stock solution**: 0.4 mg/mL of USP Ribavirin RS in Solution A

**Standard solution**: 1 µg/mL of USP Ribavirin RS in Solution A from the **Standard stock solution**

**Sample solution**: Transfer a portion of ribavirin, equivalent to 100 mg of ribavirin from finely powdered Tablets (NLT 20), to a 200-mL volumetric flask. Add about 150 mL of Solution A, and sonicate with occasional shaking for 15 min. Cool to room temperature, dilute with Solution A to volume, and mix. Pass the solution through a suitable filter of 0.45-µm pore size.

Chromatographic system
(See Chromatography (621), System Suitability.)

Mode: LC

- Detector: UV 220 nm
- Column: 4.6-mm × 25-cm; 5-µm packing L1
- Flow rate: 1 mL/min
- Injection size: 20 µL
- Run time: 70 min. [Note—Data collection is only for the first 55 min. The remaining gradient steps re-equilibrate the column.]

System suitability
Sample: Standard solution

Suitability requirements
Relative standard deviation: NMT 5.0%

Analysis

Samples: Standard solution and Sample solution

[Note—Impurities are listed in **Table 2**.]

Calculate the percentage of any unknown impurity in the portion of Tablets taken:

\[
\text{Result} = \left( \frac{r_u}{r_S} \right) \times \left( \frac{C_S}{C_U} \right) \times 100
\]

- \( r_u \) = peak response of any unknown impurity from the Sample solution
- \( r_S \) = peak response of ribavirin from the Standard solution
- \( C_S \) = concentration of USP Ribavirin RS in the Standard solution (mg/mL)
- \( C_U \) = nominal concentration of ribavirin in the Sample solution (mg/mL)

Acceptance criteria: See **Table 2**. Disregard any peak area less than 0.05%.

Published on March 26, 2020
Methyl 1-
Ribavirin methyl ester
Ribavirin 5-isomer
Ribavirin 1.0 —
Triazole acid
Triazole amide
Total impurities — 0.30
Individual unknown impurity — 0.10
Ribavirin 5'-acetyl
Ribavirin acid
-1,2,4-Triazole-3-carboxamide.
-1,2,4-Triazole-3-carboxylic acid.
-1,2,4-triazole-3-carboxylic acid.

• Organic Impurities: Procedure 2
  Buffer: 3.0 g/L of dibasic potassium phosphate in water. Adjust with phosphoric acid to a pH of 6.0 ± 0.1. Pass the solution through a suitable filter of 0.45-µm pore size.
  Mobile phase: Methanol and Buffer (1:39)
  Standard stock solution: 1 mg/mL each of USP Ribavirin RS, USP Ribavirin Related Compound A RS, USP Ribavirin Related Compound D RS, USP Uracil RS, and USP Uridine RS in water. Sonicate with occasional shaking to dissolve the solids.
  Standard solution: 0.01 mg/mL each of USP Ribavirin RS, USP Ribavirin Related Compound A RS, USP Ribavirin Related Compound D RS, USP Uracil RS, and USP Uridine RS in water from the Standard stock solution
  Sensitivity solution: 0.5 µg/mL of USP Ribavirin RS from the Standard solution in water
  Sample solution: 1.0 mg/mL. Transfer a portion of ribavirin, equivalent to 1000 mg of ribavirin from finely powdered Tablets (NLT 20), to a 1000-mL volumetric flask. Add about 500 mL of water, and sonicate with occasional shaking for 15 min. Shake the solution for 15 min, and cool to room temperature. Dilute with water to volume, and centrifuge the solution for 10 min.
  Chromatographic system
  (See Chromatography (621), System Suitability.)
  Mode: LC
  Detector: UV 207 nm
  Column: 4.6-mm × 25-cm; 5-µm packing L1
  Column temperature: 30°
  Flow rate: 1 mL/min
  Injection size: 10 µL
  Run time: NLT 4.3 times the retention time of the ribavirin peak
  System suitability
  Samples: Standard solution and Sensitivity solution
  Suitability requirements
  Signal-to-noise ratio: NLT 10, Sensitivity solution
  Relative standard deviation: NMT 2.0%, Standard solution
  Analysis
  Samples: Standard solution and Sample solution

[Note—Impurities are listed in Table 3.]
Calculate the percentage of any impurity in the portion of Tablets taken:

\[ \text{Result} = \frac{r_i}{r_s} \times \frac{C_i}{C_0} \times 100 \]

\( r_i \) = peak response of any impurity from the Sample solution
\( r_s \) = peak response of corresponding reference standard from the Standard solution
\( C_i \) = concentration of corresponding reference standard in the Standard solution (mg/mL)
\( C_0 \) = nominal concentration of ribavirin in the Sample solution (mg/mL)

Acceptance criteria: See Table 3. Disregard any peak area less than 0.05%.

Table 2

<table>
<thead>
<tr>
<th>Name</th>
<th>Relative Retention Time</th>
<th>Acceptance Criteria, NMT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triazole acids (^b)</td>
<td>0.35</td>
<td>—</td>
</tr>
<tr>
<td>Ribavirin acid (^c)</td>
<td>0.40</td>
<td>—</td>
</tr>
<tr>
<td>Triazole amide (^d)</td>
<td>0.64</td>
<td>—</td>
</tr>
<tr>
<td>Ribavirin</td>
<td>1.0</td>
<td>—</td>
</tr>
<tr>
<td>Ribavirin 5-isomer (^e)</td>
<td>1.37</td>
<td>—</td>
</tr>
<tr>
<td>Ribavirin methyl ester (^f)</td>
<td>2.09</td>
<td>—</td>
</tr>
<tr>
<td>Ribavirin 5’-acetyl (^g)</td>
<td>2.43</td>
<td>—</td>
</tr>
<tr>
<td>Ribavirin 5’-benzoyl (^h)</td>
<td>4.83</td>
<td>—</td>
</tr>
<tr>
<td>Individual unknown impurity</td>
<td>—</td>
<td>0.10</td>
</tr>
<tr>
<td>Total impurities</td>
<td>—</td>
<td>0.30</td>
</tr>
</tbody>
</table>

\( ^a \) These are process impurities listed for information only.
\( ^b \) 1H-1,2,4-Triazole-3-carboxylic acid.
\( ^c \) 1-β-D-Ribofuranosyl-1H-1,2,4-triazole-3-carboxylic acid.
\( ^d \) 1H-1,2,4-Triazole-3-carboxamide.
\( ^e \) 1-β-D-Ribofuranosyl-1H-1,2,4-triazole-5-carboxamide.
\( ^f \) Methyl 1-β-D-r-ribofuranosyl-1H-1,2,4-triazole-3-carboxylate.
\( ^g \) 1-(5-O-Acetyl-β-D-ribofuranosyl)-1H-1,2,4-triazole-3-carboxamide.
\( ^h \) 1-(5-O-Benzoyl-β-D-ribofuranosyl)-1H-1,2,4-triazole-3-carboxamide.

Ribavirin Capsules

**DEFINITION**
Ribavirin Capsules contain NLT 90.0% and NMT 110.0% of the labeled amount of ribavirin (C\(_9\)H\(_{12}\)N\(_2\)O\(_5\)).
IDENTIFICATION
• A. The retention time of the major peak of the Sample solution corresponds to that of the Standard solution, as obtained in the Assay.

ASSAY
• PROCEDURE
  Mobile phase: Water. Adjust with sulfuric acid to a pH of 2.5.
  Standard solution: 0.025 mg/mL of USP Ribavirin RS in Mobile phase
  Sample stock solution: Transfer an equivalent to 50 mg of ribavirin, from contents of Capsules (NLT 20), to a 100-mL volumetric flask. Add about 50 mL of Mobile phase, and sonicate with occasional shaking for about 20 min. Cool to room temperature, and dilute with Mobile phase to volume.
  Sample solution: Nominally 0.025 mg/mL of ribavirin in Mobile phase from Sample stock solution. Pass the solution through a suitable filter of 0.45-µm pore size.

Chromatographic system
(See Chromatography (621), System Suitability.)
Mode: LC
Detector: UV 207 nm
Column: 7.8-mm × 15-cm; 7-µm packing L17
Column temperature: 65°
Flow rate: 1 mL/min
Injection volume: 10 µL
System suitability
Sample: Standard solution
Suitability requirements
Relative standard deviation: NMT 2.0%

Analysis
Samples: Standard solution and Sample solution
Calculate the percentage of the labeled amount of ribavirin (C₈H₁₂N₄O₃) dissolved:

\[
\text{Result} = \left( \frac{r_u}{r_s} \right) \times \left( \frac{C_s}{C_u} \right) \times 100
\]

\[
r_u = \text{peak area from the Sample solution}
\]
\[
r_s = \text{peak area from the Standard solution}
\]
\[
C_s = \text{concentration of USP Ribavirin RS in the Standard solution (mg/mL)}
\]
\[
C_u = \text{nominal concentration of ribavirin in the Sample solution (mg/mL)}
\]

Acceptance criteria: 90.0%–110.0%

PERFORMANCE TESTS
• DISSOLUTION (711)
Test 1
Medium: Water; 900 mL
Apparatus 1: 100 rpm
Time: 30 min
Determine the percentage of the labeled amount of ribavirin (C₈H₁₂N₄O₃) dissolved by using one of the following procedures.

Procedure 1
Mobile phase: Proceed as directed in the Assay.
Standard solution: 22.5 µg/mL of USP Ribavirin RS in Medium
Sample solution: Pass the solution through a suitable filter of 0.45-µm pore size. Transfer 5.0 mL of the filtrate to a 50.0-mL volumetric flask, and dilute with Medium to volume.

Chromatographic system
(See Chromatography (621), System Suitability.)
Mode: LC
Detector: UV 207 nm
Column: 7.8-mm × 30-cm; 9-µm packing L17

Column temperature: 65°
Flow rate: 1.5 mL/min
Injection volume: 20 µL
System suitability
Sample: Standard solution
Suitability requirements
Relative standard deviation: NMT 2.0%

Analysis
Samples: Standard solution and Sample solution
Calculate the percentage of the labeled amount of ribavirin (C₈H₁₂N₄O₃) dissolved:

\[
\text{Result} = \left( \frac{r_u}{r_s} \right) \times \left( \frac{C_s}{L} \right) \times V \times D \times 100
\]

\[
r_u = \text{peak area from the Sample solution}
\]
\[
r_s = \text{peak area from the Standard solution}
\]
\[
C_s = \text{concentration of the Standard solution (mg/mL)}
\]
\[
L = \text{label claim (mg/Capsule)}
\]
\[
V = \text{volume of Medium, 900 mL}
\]
\[
D = \text{dilution factor of the solution under test}
\]

Procedure 2
Sulfuric acid solution: 3% sulfuric acid
Mobile phase: Water. Adjust with Sulfuric acid solution to a pH of 2.5.
Standard solution: 0.02 mg/mL of USP Ribavirin RS in Medium
Sample solution: Pass the solution through a suitable filter of 0.8-µm pore size. Transfer 5.0 mL of the filtrate to a 50.0-mL volumetric flask, and dilute with water to volume.

Chromatographic system
(See Chromatography (621), System Suitability.)
Mode: LC
Detector: UV 207 nm
Column: 7.8-mm × 10-cm; 9-µm packing L17
Column temperature: 40 ± 2°
Flow rate: 1 mL/min
Injection volume: 20 µL
System suitability
Sample: Standard solution
Suitability requirements
Tailing factor: NMT 1.5
Relative standard deviation: NMT 2.0%

Analysis
Samples: Standard solution and Sample solution
Calculate the percentage of the labeled amount of ribavirin (C₈H₁₂N₄O₃) dissolved:

\[
\text{Result} = \left( \frac{r_u}{r_s} \right) \times \left( \frac{C_s}{L} \right) \times V \times D \times 100
\]

\[
r_u = \text{peak area from the Sample solution}
\]
\[
r_s = \text{peak area from the Standard solution}
\]
\[
C_s = \text{concentration of the Standard solution (mg/mL)}
\]
\[
L = \text{label claim (mg/Capsule)}
\]
\[
V = \text{volume of Medium, 900 mL}
\]
\[
D = \text{dilution factor of the solution under test}
\]

Tolerances: NLT 80% (Q) of the labeled amount of ribavirin (C₈H₁₂N₄O₃) is dissolved.

Test 2: If the product complies with this test, the labeling indicates that the product meets USP Dissolution Test 2.
Medium: Water; 900 mL, deaerated
Apparatus 1: 100 rpm
Time: 15 min
Buffer: 4 g/L of sodium dihydrogen orthophosphate dihydrate in water. Adjust with 5% (v/v) sodium hydroxide solution to a pH of 5.0. Pass through a suitable filter of 0.45-µm or finer pore size.
Mobile phase: Acetonitrile and Buffer (2:98)
Standard solution: 0.22 mg/mL of USP Ribavirin RS in Medium. Sonicate, if necessary, to dissolve.
Sample solution: Pass a portion of the solution under test through a suitable filter of 0.45-µm pore size.

Chromatographic system
(See Chromatography (621), System Suitability.)
Mode: LC
Detector: UV 225 nm
Column: 4.6-mm × 25-cm; 5-µm packing L1
Flow rate: 1 mL/min
Injection volume: 10 µL
Run time: NLT 1.9 times the retention time of ribavirin

System suitability
Sample: Standard solution
Suitability requirements
Tailing factor: NMT 2.0
Relative standard deviation: NMT 2.0%

Analysis
Samples: Standard solution and Sample solution
Calculate the percentage of the labeled amount of ribavirin (C₆H₇N₄O₅) dissolved:

\[
\text{Result} = \left( \frac{r_u}{r_s} \right) \times \left( \frac{C_s}{C_l} \right) \times \left( \frac{1}{L} \right) \times V \times 100
\]

\( r_u \) = peak response from the Sample solution
\( r_s \) = peak response from the Standard solution
\( C_s \) = concentration of USP Ribavirin RS in the Standard solution (mg/mL)
\( L \) = label claim (mg/Capsule)
\( V \) = volume of Medium, 900 mL

Tolerances: NLT 80% (Q) of the labeled amount of ribavirin (C₆H₇N₄O₅) is dissolved.

Test 3: If the product complies with this test, the labeling indicates that the product meets USP Dissolution Test 3.

Medium: 0.1 N hydrochloric acid; 900 mL
Apparatus 1: 100 rpm
Time: 30 min

Sample solution: Pass a portion of the solution under test through a suitable filter of 0.45-µm or finer pore size.

Sample blank solution: Dissolve 6 empty Capsule shells in 900 mL of Medium. Pass through a suitable filter of 0.45-µm or finer pore size.

Instrumental conditions
Mode: UV
Analytical wavelength: UV 225 nm
Cell: 0.1 cm
Blank: Medium

Analysis
Samples: Standard solution, Sample solution, and Capsule blank solution
Calculate the percentage of the labeled amount of ribavirin (C₆H₇N₄O₅) dissolved:

\[
\text{Result} = \left( \frac{A_s - (A_b/6)}{A_l} \right) \times \left( \frac{C_s}{C_l} \right) \times \left( \frac{1}{L} \right) \times V \times 100
\]

\( A_s \) = absorbance of the Sample solution
\( A_b \) = absorbance of the Capsule blank solution
\( A_l \) = absorbance of the Standard solution
\( C_s \) = concentration of USP Ribavirin RS in the Standard solution (mg/mL)
\( L \) = label claim (mg/Capsule)
\( V \) = volume of Medium, 900 mL

Tolerances: NLT 80% (Q) of the labeled amount of ribavirin (C₆H₇N₄O₅) is dissolved.

- **Uniformity of Dosage Units (905):** Meet the requirements

**Impurities**
- **Organic impurities**

Mobile phase, Standard solution, and Chromatographic system: Proceed as directed in the Assay.

Sample solution: Nominally 0.5 mg/mL of ribavirin in Mobile phase prepared as follows. Transfer an amount equivalent to 50 mg of ribavirin, from contents of Capsules (NLT 20), to a 100-mL volumetric flask. Add about 50 mL of Mobile phase, and sonicate with occasional shaking for about 20 min. Cool to room temperature, dilute with Mobile phase to volume, and mix. Pass the solution through a suitable filter of 0.45-µm pore size.

Analysis
Samples: Standard solution and Sample solution
Calculate the percentage of ribose triazolole carboxylic acid and any other unknown impurity in the portion of Capsules taken:

\[
\text{Result} = \left( \frac{r_0}{r_s} \right) \times \left( \frac{C_s}{C_l} \right) \times \left( \frac{1}{F} \right) \times 100
\]

\( r_0 \) = peak response of ribose triazolole carboxylic acid or any other unknown impurity from the Sample solution
\( r_s \) = peak response of ribavirin from the Standard solution
\( C_s \) = concentration of USP Ribavirin RS in the Standard solution (mg/mL)
\( C_l \) = nominal concentration of ribavirin in the Sample solution (mg/mL)
\( F \) = relative response factor (see Table 1)

Acceptance criteria: See Table 1.

### Table 1

<table>
<thead>
<tr>
<th>Name</th>
<th>Relative Retention Time</th>
<th>Relative Response Factor</th>
<th>Acceptance Criteria, NMT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ribose triazolole carboxylic acid</td>
<td>0.7</td>
<td>0.7</td>
<td>0.25</td>
</tr>
<tr>
<td>Ribavirin</td>
<td>1.0</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Any individual unknown impurity</td>
<td>—</td>
<td>1.0</td>
<td>0.10</td>
</tr>
<tr>
<td>Total impurities</td>
<td>—</td>
<td>—</td>
<td>1.0</td>
</tr>
</tbody>
</table>

*a* 1-β-D-Ribofuranosyl-1H-1,2,4-triazole-3-carboxylic acid.

**Additional requirements**
- **Packaging and Storage:** Preserve in well-closed containers, and store between 15° and 30°.
- **Labeling:** When more than one Dissolution test is given, the labeling states the Dissolution test used only if Test 1 is not used.
- **USP Reference Standards (11)**
  USP Ribavirin RS

**Ribavirin for Inhalation Solution**

**Definition**
Ribavirin for Inhalation Solution is a sterile, freeze-dried form of ribavirin. When constituted as directed in the labeling, the inhalation solution so obtained contains NLT 95.0% and NMT 105.0% of the labeled amount of ribavirin (C₆H₇N₄O₅).

Published on March 26, 2020
IDENTIFICATION

Change to read:

• A. SPECTROSCOPIC IDENTIFICATION TESTS (197), Infrared Spectroscopy; 197K\* (CN 1-May-2020)

• B. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST (201)

Sample solution: 10 mg/mL
Chromatographic system
Developing solvent system: Acetonitrile and 0.1 M ammonium chloride (9:2)
Spray reagent: Anisaldehyde, alcohol, glacial acetic acid, and sulfuric acid (5:90:1.5)
Analysis: Proceed as directed in the chapter. Allow the plate to air-dry for about 15 min, spray with Spray reagent, heat the plate at 110° for 30 min, and locate the spots on the plate by examining the plate in daylight.
Acceptance criteria: Meets the requirements

ASSAY

• PROCEDURE
Mobile phase: Adjust water with sulfuric acid to a pH of 2.5 ± 0.1. Filter through a suitable filter of 0.5-µm or finer pore size.
Standard solution: 0.025 mg/mL of USP Ribavirin RS in Mobile phase
Sample stock solution: constitute Ribavirin for Inhalation Solution as directed in the labeling, using a suitable volume of diluent. Transfer an aliquot of constituted solution, equivalent to 100 mg of ribavirin, to a 200-µL volumetric flask, and dilute with Mobile phase to volume.
Sample solution: Nominally 0.025 mg/mL of ribavirin in Mobile phase from the Sample stock solution
Chromatographic system
(See Chromatography (621), System Suitability.) Mode: LC
Detector: UV 207 nm
Column: 7.8-mm x 10-cm; packing L17
Column temperature: 65 ± 0.5°
Flow rate: 1 mL/min
Injection volume: 10 µL
System suitability
Sample: Standard solution
Suitability requirements
Tailing factor: 0.7–1.5
Relative standard deviation: NMT 0.5%
Analysis
Samples: Standard solution and Sample solution
Calculate the percentage of the labeled amount of ribavirin \((C_9H_{12}N_2O_2)\) in the portion of Ribavirin for Inhalation Solution taken:

\[
\text{Result} = \left( \frac{r_u}{r_s} \right) \times \left( C_u / C_s \right) \times 100
\]

\(r_u\) = peak response from the Sample solution
\(r_s\) = peak response from the Standard solution
\(C_s\) = concentration of USP Ribavirin RS in the Standard solution (mg/mL)
\(C_u\) = nominal concentration of ribavirin in the Sample solution (mg/mL)

Acceptance criteria: 95.0%–105.0%

IMPUURIES

• RESIDUE ON IGNITION (281): NMT 0.25%

• ORGANIC IMPURITIES
Mobile phase, Standard solution, Chromatographic system, and System suitability: Proceed as directed in the Assay.

Sample solution: Prepare as directed for the Sample stock solution in the Assay.

Analysis
Sample: Sample stock solution
Calculate the percentage of each peak, other than that of the solvent peak and the ribavirin peak, in the portion of Ribavirin for Inhalation Solution taken:

\[
\text{Result} = \left( \frac{r_u}{r_s} \right) \times 100
\]

\(r_u\) = response of the individual peak
\(r_s\) = sum of all the peak responses

Acceptance criteria
Individual impurity: NMT 0.25%
Total impurities: NMT 1.0%

SPECIFIC TESTS

• STERILITY TESTS (71): Meets the requirements when tested as directed in Test for Sterility of the Product to Be Examined, Membrane Filtration.

• OPTICAL ROTATION, Specific Rotation (7815)
Sample solution: 10 mg/mL
Acceptance criteria: -33.5° to -37.0° \((t = 20°)\)

• pH (791)
Sample solution: A solution constituted as directed in the labeling. To each 50 mL of reconstituted solution add 0.2 mL of a saturated potassium chloride solution.
Acceptance criteria: 4.0–6.5

• LOSS ON DRYING (731)
Analysis: Dry a sample at 105° for 5 h.
Acceptance criteria: NMT 0.5%

ADDITIONAL REQUIREMENTS

• PACKAGING AND STORAGE: Preserve in tight containers, in a dry place at controlled room temperature.

• LABELING: The labeling indicates that Ribavirin for Inhalation Solution must be constituted with a measured volume of Sterile Water for Injection or with Sterile Water for inhalation containing no preservatives, and that the constituted solution is to be administered only by a small-particle aerosol generator.

• USP REFERENCE STANDARDS (11)
USP Ribavirin RS

Ritonavir

\[
C_{37}H_{46}N_2O_5S_2
\]
720.94
2,4,7,12-Tetraazatridecan-13-oic acid, 10-hydroxy-2-methyl-5-(1-methylthyl)-1-[2-(1-methylthyl)-4-thiazolyl]-3,6-dioxo-8,11-bis(phenylmethyl)-5-thiazolylethyl ester [5S-\((5R,8R,10R,11R)\); 5-Thiazolylethyl [[(aS)-α-1.5,3S)-1-hydroxy-3-5]-2-[3-\((2\text{-isopropyl-4-thiazolyl)methyl}-3\text{-methylureido}]3\text{-methylbutyramido}]- 4-phenylbutyl]phenethyl]carbamate [155213-67-5].

DEFINITION
Ritonavir contains NLT 97.0% and NMT 102.0% of ritonavir \((C_{37}H_{46}N_2O_5S_2)\), calculated on the anhydrous basis.

Published on March 26, 2020
IDENTIFICATION

Change to read:

• A. *SPECTROSCOPIC IDENTIFICATION TESTS* (197), Infrared Spectroscopy: 197K\textsuperscript{A} (CN 1-May-2020)
• B. The retention time of the major peak of the Sample solution is within 2% of the retention time of the major peak of the Standard solution, as obtained in the Assay.

ASSAY

• PROCEDURE

Solution A: 4.1 mg/mL of monobasic potassium phosphate in water

Solution B: Acetonitrile, tetrahydrofuran (inhibitor-free), n-butanol, and Solution A (18:8:5:69)

Mobile phase: Solution B

Diluent: Acetonitrile and Solution A (1:1)

Standard stock solution: 2.0 mg/mL of USP Ritonavir RS in Diluent. [Note—This solution may be kept for 5 days if refrigerated.]

Standard solution 1: 0.10 mg/mL of USP Ritonavir RS from the Standard stock solution diluted with Diluent

Standard solution 2: 0.025 mg/mL of USP Ritonavir RS from Standard solution 1 diluted with Diluent

Sample solution: 0.025 mg/mL of USP Ritonavir in Diluent

Chromatographic system

(See Chromatography (621), System Suitability.)

Mode: LC

Detector: UV 240 nm

Column: 4.6-mm × 15-cm; 3-µm packing L26

Column temperature: 60°

Flow rate: 1 mL/min

Injection volume: 50 µL

Run time: 40 min

System suitability

Sample: Standard solution 2

Suitability requirements

Capacity factor, \( k' \): NLT 13

Column efficiency: NLT 5000 theoretical plates

Tailing factor: 0.8–1.2

Relative standard deviation: NMT 3.0%

Analysis

Samples: Standard solution 2 and Sample solution

Calculate the percentage of ritonavir (C\textsubscript{4}H\textsubscript{11}N\textsubscript{2}O\textsubscript{3}S\textsubscript{2}) in the portion of Ritonavir taken:

\[
\text{Result} = \left( \frac{r_U}{r_S} \right) \times \left( \frac{C_S}{C_U} \right) \times 100
\]

where:

- \( r_U \) = peak response from the Sample solution
- \( r_S \) = peak response from the Standard solution
- \( C_S \) = concentration of USP Ritonavir RS in Standard solution 2 (mg/mL)
- \( C_U \) = concentration of Ritonavir in the Sample solution (mg/mL)

Acceptance criteria: 97.0%–102.0% on the anhydrous basis

IMPURITIES

• RESIDUE ON IGNITION (281): NMT 0.2%, determined on 1.0 g

• ORGANIC IMPURITIES

Ritonavir is alkali sensitive. All glassware should be prerinsed with distilled water before use to remove residual detergent contamination.


Solution C: Acetonitrile, tetrahydrofuran (inhibitor-free), n-butanol, and Solution A (47:8:5:40)

Mobile phase: See Table 1.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Solution B (%)</th>
<th>Solution C (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>60</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>120</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>120.1</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>155</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

Identity solution: 1 mg/mL of USP Ritonavir Related Compounds Mixture RS in Diluent

Standard solution 1: 5 µg/mL of USP Ritonavir RS from Standard solution 1 in Diluent. [Note—This is stable for 48 h.]

Sample solution: 1 mg/mL of Ritonavir in Diluent

Chromatographic system

(See Chromatography (621), System Suitability.)

Mode: LC

Detector: UV 240 nm

Column: 4.6-mm × 15-cm; 3-µm packing L26

Column temperature: 60°

Flow rate: 1 mL/min

Injection volume: 50 µL

Run time: Standard solution 2: 40 min

System suitability

Samples: Identity solution and Standard solution 2

[Note—See Table 2 for relative retention times.]

Suitability requirements

Resolution: NLT 1.0 between hydroxyritonavir and hydantoin aminoalcohol peaks, Identity solution

Peak-to-valley ratio: NLT 1 for ritonavir and the 4-hydroxy isomer, Identity solution

Capacity factor, \( k' \): NLT 13, Standard solution 2

Column efficiency: NLT 5000 theoretical plates, Standard solution 2

Tailing factor: 0.8–1.2, Standard solution 2

Relative standard deviation: NMT 3.0%, Standard solution 2

Analysis

Samples: Diluent, Identity solution, Standard solution 2, and Sample solution

Calculate the percentage of each impurity in the portion of Ritonavir taken:

\[
\text{Result} = \left( \frac{r_U}{r_S} \right) \times \left( \frac{C_S}{C_U} \right) \times \left( \frac{1}{F} \right) \times 100
\]

where:

- \( r_U \) = peak response of each impurity from the Sample solution
- \( r_S \) = peak response from Standard solution 2
- \( C_S \) = concentration of Standard solution 2 (mg/mL)
- \( C_U \) = concentration of Ritonavir in the Sample solution (mg/mL)
- \( F \) = relative response factor (see Table 2)

Acceptance criteria: See Table 2.

<table>
<thead>
<tr>
<th>Name</th>
<th>Retention Time (min)</th>
<th>Relative Response Factor</th>
<th>Acceptance Criteria, NMT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mixture of ureidovaline and N-deacylvaline ritonavir*</td>
<td>0.07</td>
<td>1.0</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Published on March 26, 2020
### Table 2 (continued)

<table>
<thead>
<tr>
<th>Name</th>
<th>Relative Retention Time</th>
<th>Relative Response Factor</th>
<th>Acceptance Criteria, NMT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetamidoalcohol&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.15</td>
<td>1.0</td>
<td>0.1</td>
</tr>
<tr>
<td>2,5-Thiazolylmethyl dicarbamate&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.24</td>
<td>1.37</td>
<td>0.1</td>
</tr>
<tr>
<td>Hydroxyl ritonavir&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.36</td>
<td>1.0</td>
<td>0.3</td>
</tr>
<tr>
<td>Hydantoin aminoalcohol&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.39</td>
<td>0.73</td>
<td>0.1</td>
</tr>
<tr>
<td>Ritonavir hydperoxide&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.45</td>
<td>1.0</td>
<td>0.1</td>
</tr>
<tr>
<td>Hydantoin-oxazolidinone derivative&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.47</td>
<td>0.76</td>
<td>0.1</td>
</tr>
<tr>
<td>Ethyl analog&lt;sup&gt;g&lt;/sup&gt;</td>
<td>0.64</td>
<td>1.0</td>
<td>0.1</td>
</tr>
<tr>
<td>Mixture of BOC-aminoalcohol and isobutoxycarboxyl aminoalcohol&lt;sup&gt;h&lt;/sup&gt;</td>
<td>0.81</td>
<td>0.74</td>
<td>0.1</td>
</tr>
<tr>
<td>Oxazolidinone derivative&lt;sup&gt;i&lt;/sup&gt;</td>
<td>0.87</td>
<td>0.53</td>
<td>0.1</td>
</tr>
<tr>
<td>Ureidovaline isobutyl ester&lt;sup&gt;j&lt;/sup&gt;</td>
<td>0.94</td>
<td>1.0</td>
<td>0.1</td>
</tr>
<tr>
<td>4-Hydroxy isomer&lt;sup&gt;k&lt;/sup&gt;</td>
<td>1.05</td>
<td>1.0</td>
<td>0.1</td>
</tr>
<tr>
<td>3R-Epimer&lt;sup&gt;l&lt;/sup&gt;</td>
<td>1.11</td>
<td>1.0</td>
<td>0.3</td>
</tr>
<tr>
<td>Aminoalcohol urea derivative&lt;sup&gt;m&lt;/sup&gt;</td>
<td>1.14</td>
<td>1.0</td>
<td>0.1</td>
</tr>
<tr>
<td>3R,5R-Diastereomer&lt;sup&gt;n&lt;/sup&gt;</td>
<td>1.23</td>
<td>1.0</td>
<td>0.1</td>
</tr>
<tr>
<td>5R-Epimer&lt;sup&gt;o&lt;/sup&gt;</td>
<td>1.32</td>
<td>1.0</td>
<td>0.1</td>
</tr>
<tr>
<td>Diacyl valine urea&lt;sup&gt;p&lt;/sup&gt;</td>
<td>1.62</td>
<td>1.0</td>
<td>0.1</td>
</tr>
<tr>
<td>Divalinyl analog&lt;sup&gt;q&lt;/sup&gt;</td>
<td>2.87</td>
<td>0.73</td>
<td>0.2</td>
</tr>
<tr>
<td>O-Acyl ritonavir&lt;sup&gt;r&lt;/sup&gt;</td>
<td>3.20</td>
<td>1.0</td>
<td>0.1</td>
</tr>
<tr>
<td>Any other individual impurity</td>
<td>—</td>
<td>1.0</td>
<td>0.1</td>
</tr>
</tbody>
</table>

<sup>a</sup> Ureidovaline is [N-methyl(2-isopropyl-4-thiazolyl)methyl]amino[carboxyl-L-valine and N-deaclyvaline ritonavir is thiazol-5-yymethyl (2S,3S,5S)-S(3S)-3-amino-3-methylbutanamido]-3-hydroxy-1,6-diphenyloxan-2-yl carbamate.

<sup>b</sup> Thiazol-5-yymethyl (2S,3S,5S)-S(3S)-3-acetamido-3-hydroxy-1,6-diphenyloxan-2-yl carbamate.

<sup>c</sup> Bis(thiazol-5-yymethyl) (2S,3S,5S)-S(3S)-3-hydroxy-1,6-diphenyloxan-2-yl carbamate.

<sup>d</sup> Thiazol-5-yymethyl (2S,3S,5S)-S(3S)-3-hydroxy-1,6-diphenyloxan-2-yl carbamate.

<sup>e</sup> Hydroxyritonavir.

<sup>f</sup> Derivative.

<sup>g</sup> Isobutyl analog.

<sup>h</sup> Mixture of BOC-aminoalcohol and isobutoxycarboxyl aminoalcohol.

<sup>i</sup> Oxazolidinone derivative.

<sup>j</sup> Ureidovaline isobutyl ester.

<sup>k</sup> 4-Hydroxy isomer.

<sup>l</sup> 3R-Epimer.

<sup>m</sup> Aminoalcohol urea derivative.

<sup>n</sup> 3R,5R-Diastereomer.

<sup>o</sup> 5R-Epimer.

<sup>p</sup> Diacyl valine urea.

<sup>q</sup> Divalinyl analog.

<sup>r</sup> O-Acyl ritonavir.

<sup>s</sup> Any other individual impurity.

### Specific Tests

- **Water Determination**: Method II: NMT 0.5%, determined on 0.500 g.

### Additional Requirements

- **Packaging and Storage**: Preserve in tight, light-resistant containers. Store between 5° and 30°.

### USP Reference Standards

- USP Ritonavir RS
- USP Ritonavir Related Compounds Mixture RS

### Ritonavir Capsules

**Definition**

Ritonavir Capsules contain NLT 90.0% and NMT 110.0% of the labeled amount of ritonavir (C₇₇H₆₅N₅O₁₃S₂).
IDENTIFICATION

• A. The retention time of the major peak of the Sample solution corresponds to that of the Standard solution, as obtained in the Assay.

ASSAY

• Procedure
Buffer: 4.1 g/L of monobasic potassium phosphate
Diluent: Acetonitrile and Buffer (50:50)
Mobile phase: Acetonitrile, methanol, tetrahydrofuran (stabilizer-free), and Buffer (7:4:4:25). Separately filter the Buffer and the pre-mixed solvents before combining to make the Mobile phase.

Standard solution: 25 µg/mL of USP Ritonavir RS in Diluent
Sample stock solution: Nominally 1 mg/mL of ritonavir prepared as follows. Transfer Capsules (NLT 5) equivalent to 500 mg of ritonavir into a 500-mL volumetric flask, add about 250 mL of Diluent, and shake for at least 30 min or until the Capsules have visually disintegrated. Add 150 mL of acetonitrile, allow to cool to room temperature, and dilute to volume with Diluent.
Sample solution: Nominally 25 µg/mL of ritonavir in Diluent from the Sample stock solution

Chromatographic system
(See Chromatography (621), System Suitability.)
Mode: LC
Detector: UV 240 nm
Column: 4.6-mm × 15-cm; 5-µm packing L7
Column temperature: 40°C
Flow rate: 1.5 mL/min
Injection volume: 50 µL

System suitability
Sample: Standard solution
Suitability requirements
Capacity factor: NMT 2.0%
Tailing factor: NMT 1.5
Relative standard deviation: NMT 2.0%

Analysis
Samples: Standard solution and Sample solution
Calculate the percentage of the labeled amount of ritonavir (C_{17}H_{24}N_{6}O_{5}S_{2}) dissolved:

\[ \text{Result} = \left( \frac{r_0}{r_i} \right) \times \left( \frac{C_i}{C_0} \right) \times V \times 100 \]

\[ C_0 = \text{peak response from the Sample solution} \]
\[ r_0 = \text{peak response from the Standard solution} \]
\[ C_i = \text{concentration of USP Ritonavir RS in the Standard solution (mg/mL)} \]
\[ L = \text{ritonavir label claim (mg/Capsule)} \]
\[ V = \text{volume of Medium, 900 mL} \]

Tolerances: NLT 80% (Q) of the labeled amount of ritonavir (C_{17}H_{24}N_{6}O_{5}S_{2}) is dissolved.

*Test 2: If the product complies with this test, the labeling indicates that it meets USP Dissolution Test 2.
Medium: 0.1 N hydrochloric acid with 25 mM polyoxyethylene 10 lauryl ether; 900 mL, degassed
Apparatus 2: 50 rpm, with sinker
Time: 20 and 120 min
Solution A: Water and phosphoric acid (98:2)
Buffer: Water adjusted with Solution A to a pH of 3.5
Mobile phase: Acetonitrile, methanol, and Buffer (500:100:400)
Standard stock solution: 0.56 mg/mL of USP Ritonavir RS in methanol
Standard solution: 0.11 mg/mL of USP Ritonavir RS in Medium from Standard stock solution
Sample solution: Pass a portion of the solution under test through a suitable filter of 0.45-µm pore size.

Chromatographic system
(See Chromatography (621), System Suitability.)
Mode: LC
Detector: UV 240 nm
Column: 4.6-mm × 15-cm; 5-µm packing L7
Column temperature: 30°C
Flow rate: 1.8 mL/min
Injection volume: 20 µL

System suitability
Sample: Standard solution
Suitability requirements
Tailing factor: NMT 2.0
Relative standard deviation: NMT 2.0%

Analysis
Samples: Standard solution and Sample solution
Calculate the concentration (C) of ritonavir (C_{17}H_{24}N_{6}O_{5}S_{2}) in the sample withdrawn from the vessel at each time point (i):

\[ \text{Result}_i = \left( \frac{r_0}{r_i} \right) \times C_i \]

Published on March 26, 2020
Calculate the percentage of the labeled amount of ritonavir \( (\text{C}_7\text{H}_{14}\text{N}_{10}\text{O}_{3}\text{S}) \) dissolved at each time point \( i \):

\[
\text{Result}_i = \text{C}_i \times V \times (1/L) \times 100
\]

\[
\text{Result}_s = \left( \left( \text{C}_s \times (V - V_b) \right) + (\text{C}_s \times V_b) \right) \times (1/L) \times 100
\]

\[\text{C}_i = \text{concentration of ritonavir in the portion of sample withdrawn at the specified time point } i \text{ (mg/mL)}\]

\[V = \text{volume of Medium, 900 mL}\]

\[L = \text{label claim of ritonavir (mg/Capsule)}\]

\[V_b = \text{volume of the Sample solution withdrawn at each time point } i \text{ (mL)}\]

**Tolerances:** See Table 1.

### Table 1

<table>
<thead>
<tr>
<th>Time Point (i)</th>
<th>Time (min)</th>
<th>Tolerances *</th>
<th>▲</th>
<th>▲</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20</td>
<td>20%−40%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>120</td>
<td>NLT 80%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### IMPURITIES

**Organic Impurities**

Note—Ritonavir is alkali sensitive. All glassware should be pre-rinsed with distilled water before use to remove residual detergent contaminant.

**Buffer A:** 4.1 g/L of monobasic potassium phosphate

**Buffer B:** 3.8 g/L of monobasic potassium phosphate and 0.25 g/L of dibasic potassium phosphate

**Solution A:** Acetonitrile and Buffer A (50:50)

**Solution B:** Acetonitrile and Buffer A (65:35)

**Solution C:** Butyl alcohol and Buffer A (8:92)

**Mobile phase:** Acetonitrile, butyl alcohol, tetrahydrofuran (stabilizer-free), and Buffer B (18:5:8:69), Adjust apparent pH to 6.3 ± 0.1 with 1 M phosphoric acid or 1 M potassium hydroxide if necessary.

**Cleaning solution:** Acetonitrile, butyl alcohol, tetrahydrofuran (stabilizer-free), and Buffer A (30:8:13:49)

**Standard stock solution:** 0.1 mg/mL of USP Ritonavir RS in Solution A

**Standard solution:** 10 µg/mL of USP Ritonavir RS in Solution C from Standard stock solution

**Peak identification solution:** Transfer 5–10 g from contents of Capsules into a suitable sealed container. Add an amount of citric acid equivalent to 1% of the Capsule weight taken, and mix until dissolved. Seal the container, and heat at 60° for about 24 h. Transfer about 2 g to a 100-mL volumetric flask, and dilute with Solution B to volume. Transfer 5.0 mL of the solution to a 50-mL centrifuge tube that has been previously rinsed with methanol and dried. Add 20.0 mL of heptane, and seal the tube with a stopper. Shake vigorously until a uniform emulsion is obtained, making sure to vent periodically. The emulsion formed yields distinct layers when centrifuged. The top layer (clear heptane) and the bottom layer (clear sample solution) are separated by a viscous white cloudy layer. The middle layer is part of the heptane layer. Carefully remove the clear heptane layer and the middle layer. Pass the bottom layer through a solid phase extraction cartridge containing strong anion-exchange packing in acetate form as described below.

**Sample stock solution:** Nominally 2 mg/mL of ritonavir prepared as follows. Empty the contents of Capsules (NLT 6) into a suitable container, and accurately weigh and transfer an equivalent to 200 mg of ritonavir to a 100-mL volumetric flask. Dissolve and dilute with Solution B to volume.

**Sample solution:** Nominally 1 mg/mL of ritonavir prepared as follows. Transfer 25.0 mL of Sample stock solution into a 50-mL volumetric flask, and dilute with Solution C to volume. Add 15.0 mL of this solution into a 50-mL centrifuge tube that has been previously rinsed with methanol and dried. Add 20.0 mL of heptane, and seal the tube with a stopper. Shake vigorously until a uniform emulsion is obtained, making sure to vent periodically. The emulsion formed yields distinct layers when centrifuged. The top layer (clear heptane) and the bottom layer (clear sample solution) are separated by a viscous white cloudy layer. The middle layer is part of the heptane layer. Carefully remove the clear heptane layer and the middle layer. Pass the bottom layer through a solid phase extraction cartridge containing strong anion-exchange packing in acetate form as described below.

Condition a solid phase extraction cartridge with methanol and Solution B two separate times, and dry for 10 min under low vacuum. Add 5.0 mL of the clear sample solution into the reservoir. Collect the sample solution at a slow rate into a 5-mL volumetric flask using low vacuum. Dilute with Solution B to volume.

**Chromatographic system**

(See Chromatography (621), System Suitability.)

**Mode:** LC

**Detector:** UV 240 nm

**Column:** 4.6-mm × 15-cm; 3-µm packing L26. Wash the column after each injection of the Peak identification solution and each injection of the Sample solution with Cleaning solution for about 26 min, and equilibrate with Mobile phase for about 30 min. Store in Cleaning solution after the analysis is completed.

**Column temperature:** 60°

**Flow rate:** 1 mL/min

**Injection volume:** 50 µL

**Run time:** 1.8 times the retention time of ritonavir

**System suitability**

**Sample:** Standard solution

**Suitability requirements**

**Capacity factor:** NLT 13

**Tailing factor:** 0.8−1.2

**Relative standard deviation:** NMT 3.0%

**Analysis**

**Samples:** Peak identification solution, Standard solution, and Sample solution

Calculate the percentage of each impurity in the portion of Capsules taken:

\[
\text{Result} = \left( \frac{r_S}{r_i} \right) \times \left( \frac{C_i}{C_0} \right) \times (1/F) \times 100
\]

\[r_i = \text{peak response of each impurity from the Sample solution}\]

\[r_S = \text{peak response of ritonavir from the Standard solution}\]

\[C_i = \text{concentration of USP Ritonavir RS in the Standard solution (mg/mL)}\]
**SPECIFIC TESTS**

- **Microbial Enumeration Tests** (61) and Tests for Specified Microorganisms (62): The total aerobic microbial count does not exceed 10² cfu/g. It meets the requirements of the test for absence of Escherichia coli and Salmonella.

**ADDITIONAL REQUIREMENTS**

- **Packaging and Storage:** Preserve in tight, light-resistant containers. Store between 2° and 8°.

**Add the following:**

**Labeling:** When more than one Dissolution test is given, the labeling states the Dissolution test used only if Test 1 is not used.  

**Published on March 26, 2020**
Ritonavir Tablets

DEFINITION
Ritonavir Tablets contain NLT 90.0% and NMT 110.0% of the labeled amount of ritonavir (C_{31}H_{48}N_{6}O_{5}S_{2}).

IDENTIFICATION
• A. The retention time of the major peak of the Sample solution corresponds to that of the Standard solution, as obtained in the Assay.

ASSAY
• PROCEDURE
Buffer: 4.1 g/L of monobasic potassium phosphate
Solution A: Acetonitrile and Buffer (50:50)
Solution B: Acetonitrile, butyl alcohol, water, and Buffer (65:15:10:10)
Mobile phase: Acetonitrile, methanol, tetrahydrofuran (stabilizer-free), and Buffer (17.5:10:10:62.5). Filter the required solutions individually before use.
Standard solution: 0.1 mg/mL of USP Ritonavir RS in Solution A
Sample stock solution: Nominally 1 mg/mL of ritonavir prepared as follows. Transfer Tablets (NLT 5) equivalent to 200 mg of ritonavir into a 500-mL volumetric flask. Fill the flask half full with Solution B, and mechanically shake for at least 60 min or until the Tablets have visually disintegrated. Dilute with Solution B to volume, and stir for 30 min. Transfer a sufficient quantity of this solution to a centrifuge tube, and centrifuge for about 15 min. Use the supernatant to prepare the Sample solution.
Sample solution: Nominally 0.1 mg/mL of ritonavir in Solution A from the supernatant of Sample stock solution

Chromatographic system
(See Chromatography (621), System Suitability.)
Mode: LC
Detector: UV 215 nm
Column: 4.6-mm × 15-cm; 5-µm packing, L7
Column temperature: 40°C
Flow rate: 1.5 mL/min
Injection volume: 50 µL
System suitability
Sample: Standard solution
Suitability requirements
Tailing factor: 0.9–1.5
Capacity factor: Greater than 3.5
Relative standard deviation: NMT 2.0%

Analysis
Samples: Standard solution and Sample solution
Calculate the percentage of the labeled amount of ritonavir (C_{31}H_{48}N_{6}O_{5}S_{2}) dissolved:

Result = \( \frac{r_u}{r_s} \times \frac{(C_s/C_u)}{V} \times 100 \)

where:
- \( r_u \) = peak response from the Sample solution
- \( r_s \) = peak response from the Standard solution
- \( C_s \) = concentration of USP Ritonavir RS in the Standard solution (mg/mL)
- \( C_u \) = nominal concentration of ritonavir in the Sample solution (mg/mL)
- \( V \) = volume of Medium, 900 mL

Tolerances: NLT 75% (Q) of the labeled amount of ritonavir (C_{31}H_{48}N_{6}O_{5}S_{2}) is dissolved.

• IMPURITIES

Organic impurities
Ritonavir is alkali sensitive. All glassware should be pre-rinsed with distilled water before use to remove residual detergent contamination.
Buffer A: 4.1 g/L of monobasic potassium phosphate
Buffer B: 3.8 g/L of dibasic potassium phosphate
Buffer C: Acetonitrile and Buffer B (50:50)
Solution A: Acetonitrile, butyl alcohol, water, and Buffer A (65:15:10:10)
Solution B: Acetonitrile, butyl alcohol, and Buffer A (15:5:80)
Mobile phase: Acetonitrile, butyl alcohol, tetrahydrofuran (stabilizer-free), and Buffer B (18:5:8:69) adjusted with 1 M phosphoric acid or 1 M potassium hydroxide, if necessary, to an apparent pH of 6.3 ± 0.1
Cleaning solution: Acetonitrile, butyl alcohol, tetrahydrofuran (stabilizer-free), and Buffer A (30:8:13:49)
Standard stock solution: 2.5 µg/mL of USP Ritonavir RS in Solution C from Standard stock solution
System suitability stock solution: 1 mg/mL of USP Ritonavir Related Compounds Mixture RS in Solution B

System suitability solution: 0.5 mg/mL of USP Ritonavir Related Compounds Mixture RS in Solution C from System suitability stock solution

Sample stock solution: Nominally 1 mg/mL prepared as follows. Transfer Tablets (NLT 5) equivalent to 500 mg of ritonavir into a 500-mL volumetric flask. Fill the flask half full with Solution B, and mechanically shake for at least 60 min or until the Tablets have visually disintegrated. Dilute with Solution B to volume, and stir for 30 min. Transfer a sufficient quantity of this solution to a centrifuge tube, and centrifuge for 15 min. Use the supernatant to prepare the Sample solution.

Sample solution: Nominally 0.5 mg/mL of ritonavir in Solution C from the supernatant of Sample stock solution

Chromatographic system
(See Chromatography (621), System Suitability.)
Mode: LC
Detector: UV 240 nm
Column: 4.6-mm × 15-cm; 3-µm packing L26. Wash the column after each injection of the Sample solution with Cleaning solution for about 26 min, and equilibrate with Mobile phase for about 30 min. Store in Cleaning solution after the analysis is completed.

Column temperature: 60°
Flow rate: 1 mL/min
Injection volume: 50 µL
Run time: 2.4 times the retention time of ritonavir

System suitability
Samples: Standard solution and System suitability solution
See Table 1 for relative retention values. Disregard all peaks occurring before the N-deacetylvaline ritonavir peak.

Suitability requirements
Resolution: Greater than 0.7 between the hydroxyritonavir and hydantoin aminoalcohol peaks, System suitability solution
Capacity factor: Greater than 10.8, Standard solution
Tailing factor: 0.8–1.2, Standard solution
Relative standard deviation: NMT 5.0%, Standard solution

Table 1 (continued)

<table>
<thead>
<tr>
<th>Name</th>
<th>Relative Retention</th>
<th>Relative Response Factor</th>
<th>Acceptance Criteria, NMT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,5-Thiazolymethylidicarbamate&lt;sup&gt;h&lt;/sup&gt;</td>
<td>0.24</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Hydroxyritonavir&lt;sup&gt;h&lt;/sup&gt;</td>
<td>0.36</td>
<td>1.0</td>
<td>0.3</td>
</tr>
<tr>
<td>Hydantoin aminoalcohol&lt;sup&gt;h&lt;/sup&gt;</td>
<td>0.39</td>
<td>0.73</td>
<td>2.6</td>
</tr>
<tr>
<td>Ritonavir hydroperoxide&lt;sup&gt;h&lt;/sup&gt;</td>
<td>0.44</td>
<td>1.0</td>
<td>0.2</td>
</tr>
<tr>
<td>Hydantoin-oxazolidinone derivative&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.50</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Ethyl analog&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.64</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Geo-isomer&lt;sup&gt;h&lt;/sup&gt;</td>
<td>0.74</td>
<td>1.0</td>
<td>0.2</td>
</tr>
<tr>
<td>BOC-aminoalcohol&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.81</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Isobutoxycarbonyl aminoalcohol&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.87</td>
<td>0.53</td>
<td>0.3</td>
</tr>
<tr>
<td>Oxazolidinone derivative&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.87</td>
<td>0.53</td>
<td>0.3</td>
</tr>
<tr>
<td>Ureidovaline isobutyl ester&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.94</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Ritonavir</td>
<td>1.00</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>4-Hydroxy isomer&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1.05</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>3R-Epimer&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1.11</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Aminoalcohol urea derivative&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1.14</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>3R,5SR-Diastereomer&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1.23</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>5R-Epimer&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1.32</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Diacyl valine urea&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1.70</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Any individual unspecified degradation product</td>
<td>—</td>
<td>1.0</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Acceptance criteria: See Table 1. Disregard peaks less than 0.05%.

Analysis
Samples: Standard solution and Sample solution
Calculate the percentage of each impurity in the portion of Tablets taken:

\[ \text{Result} = \left( \frac{r_0}{r_s} \right) \times \left( \frac{C_s}{C_U} \right) \times \left( \frac{1}{F} \right) \times 100 \]

\( r_0 \) = peak response of each impurity from the Sample solution
\( r_s \) = peak response of ritonavir from the Standard solution
\( C_s \) = concentration of USP Ritonavir RS in the Standard solution (mg/mL)
\( C_U \) = nominal concentration of ritonavir in the Sample solution (mg/mL)
\( F \) = relative response factor (See Table 1)

Acceptance criteria: See Table 1. Disregard peaks less than 0.05%.

Table 1

<table>
<thead>
<tr>
<th>Name</th>
<th>Relative Retention</th>
<th>Relative Response Factor</th>
<th>Acceptance Criteria, NMT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Deacetylvaline ritonavir&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.11</td>
<td>1.0</td>
<td>0.2</td>
</tr>
<tr>
<td>Acetamidoalcohol&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.13</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>
**Table 1 (continued)**

<table>
<thead>
<tr>
<th>Name</th>
<th>Relative Retention</th>
<th>Relative Response Factor</th>
<th>Acceptance Criteria, NMT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total impurities</td>
<td>—</td>
<td>—</td>
<td>3.5</td>
</tr>
</tbody>
</table>

a Thiazol-5-ylmethyl (2S,3S,5S)-5-((S)-2-amino-3-methylbutanamido)-3-hydroxy-1,6-diphenylhexan-2-ylcarbamate.
b Degradation product.
c Thiazol-5-ylmethyl (2S,3S,5S)-5-acetamido-3-hydroxy-1,6-diphenylhexan-2-ylcarbamate.
d Process impurity included in this table for peak identification only. This impurity is controlled in the drug substance. It is not to be reported for the drug product nor included in the total impurities.
e Bis(thiazol-5-ylmethyl) (2S,3S,5S)-3-hydroxy-1,6-diphenylhexan-2-sydlydicarbamate. (Two peaks may be detected with a relative retention value of 0.24. The first peak is considered as an unknown impurity and the second as 2,5-thiaryl methyl dicarbamate.)
f Thiazol-5-ylmethyl (2S,3S,5S)-3-hydroxy-5-((S)-2-((2-hydroxypropan-2-yl)thiazol-4-yl)methyl)-3-methylbutanamido)-1,6-diphenylhexan-2-ylcarbamate.
g Thiazol-5-ylmethyl (2S,3S,5S)-3-hydroxy-5-((S)-4-isopropyl-2,5-dioximidazolidin-1-yl)-1,6-diphenylhexan-2-ylcarbamate.
h Thiazol-5-ylmethyl (2S,3S,5S)-5-((S)-2-((2-(2-hydroxypropan-2-yl)thiazol-4-yl)methyl)-3-methylbutanamido)-3-hydroxy-1,6-diphenylhexan-2-ylcarbamate.
i (4S,5S)-Thiazol-5-ylmethyl 4-benzyl-5-((S)-2-((4-isopropyl-2,5-dioximidazolidin-1-yl)-3-phenylpropyl)-2-oxooxazolidine-3-carboxylate.
j Thiazol-5-ylmethyl (2S,3S,5S)-5-((S)-2-3-((2-ethylthiazol-4-yl)methyl)-3-methylbutanamido)-3-methylbutanamido)-3-hydroxy-1,6-diphenylhexan-2-ylcarbamate.
k Thiazol-5-ylmethyl (2S,3S,5S)-5-Amino-1,6-diphenyl-2-((thiazol-5-ylmethyl)carbonylarnino)hexan-3-yl)-3-(3-(2-isopropylthiazol-4-yl)methyl)-3-methylbutanamido).
l Thiazol-5-ylmethyl (2S,3S,5S)-5-butyroxy carbonylamino)-3-hydroxy-1,6-diphenylhexan-2-ylcarbamate. (May co-elute with isobutoxycarbonyl aminolcohol).
m Thiazol-5-ylmethyl (2S,3S,5S)-5-isobutoxycarbonyl amino)-3-hydroxy-1,6-diphenylhexan-2-ylcarbamate. (May co-elute with BOC-aminoalcohol).
n (S)-(N-((S)-(1-((4S,5S)-4-Benzyl-2-oxooxazolidin-5-yl)-3-phenylpropan-2-yl)-2-(3-(2-isopropylthiazol-4-yl)methyl)-3-methylbutanamido).
o (S)-Isobutyl 2-(3-((2-isopropylthiazol-4-yl)methyl)-3-methylbutanamido).
p Thiazol-5-ylmethyl (2S,4S,5S)-4-hydroxy-5-((S)-2-3-(2-isopropylthiazol-4-yl)methyl)-3-methylbutanamido).
q Thiazol-5-ylmethyl (2S,3R,5S)-3-hydroxy-5-((S)-2-3-(2-isopropylthiazol-4-yl)methyl)-3-methylbutanamido)
• 1,6-diphenylhexan-2-ylcarbamate.
r Thiazol-5-ylmethyl (2S,3R,5S)-3-hydroxy-5-((S)-2-3-(2-isopropylthiazol-4-yl)methyl)-3-methylbutanamido)
• 1,6-diphenylhexan-2-ylcarbamate.
s Bis(thiazol-5-ylmethyl) (2S,5S)-3,5,5'-2,5'-carbonylbis(azanediyl)
• 3-hydroxy-1,6-diphenylhexane-5,2-diyldicarbamate.
t Thiazol-5-ylmethyl (2S,3R,5R)-3-hydroxy-5-((S)-2-3-(2-isopropylthiazol-4-yl)methyl)-3-methylbutanamido)
• 1,6-diphenylhexan-2-ylcarbamate.
u Thiazol-5-ylmethyl (2S,3S,5R)-3-hydroxy-5-((S)-2-3-(2-isopropylthiazol-4-yl)methyl)-3-methylbutanamido)
• 1,6-diphenylhexan-2-ylcarbamate.

ADDITIONAL REQUIREMENTS

a Packaging and Storage: Preserve in tight containers. Store at or below 30°C.
b USP Reference Standards (11)

Ritonavir Oral Solution

**DEFINITION**

Ritonavir Oral Solution contains NLT 90.0% and NMT 110.0% of the labeled amount of ritonavir (C29H34N4O4S2).

**IDENTIFICATION**

- A. The retention time of the major peak of the Sample solution corresponds to that of the Standard solution, as obtained in the Assay.

**ASSAY**

- **PROCEDURE**

Buffer: 4.1 g/L of monobasic potassium phosphate

Mobile phase: Acetonitrile, methanol, tetrahydrofuran (stabilizer-free), and Buffer (17.5: 10: 10: 62.5). Filter the required solutions individually prior to use.

Diluent: Acetonitrile and Buffer (50:50)

Standard solution: 25 µg/mL of USP Ritonavir RS in Diluent

Sample solution: Nominally 25 µg/mL of ritonavir in Diluent from a measured volume of Oral Solution

**Chromatographic system**

(See Chromatography (621), System Suitability.)

**Mode:** LC

**Detector:** UV 240 nm

**Column:** 4.6-mm x 15-cm; S-µm packing L7

**Column temperature:** 40°C

**Flow rate:** 1.5 mL/min

**Injection volume:** 50 µL

**System suitability**

Sample: Standard solution

Suitability requirements

- Tailing factor: 0.8–1.2
- Relative standard deviation: NMT 2.0%

**Analysis**

Samples: Standard solution and Sample solution

Calculate the percentage of the labeled amount of ritonavir (C29H34N4O4S2) in the portion of Oral Solution taken:

\[ \text{Result} = \left( \frac{t_0}{t_i} \right) \times \left( \frac{C_i}{C_j} \right) \times 100 \]

where:

- \( t_0 \) = peak response from the Sample solution
- \( t_i \) = peak response from the Standard solution
- \( C_i \) = concentration of USP Ritonavir RS in the Standard solution (µg/mL)
- \( C_j \) = nominal concentration of ritonavir in the Sample solution (µg/mL)

**Acceptance criteria:** 90.0%–110.0%

**PERFORMANCE TESTS**

- **DELIVERABLE VOLUME (698)**

For multiple-unit containers

Acceptance criteria: Meets the requirements

**IMPURITIES**

- **ORGANIC IMPURITIES**

  \[ \text{NOT}–\text{Ritonavir is alkali sensitive. All glassware should be prerinsed with distilled water before use to remove residual detergent contamination.} \]

  Buffer A: 4.1 g/L of monobasic potassium phosphate

  Buffer B: 3.8 g/L of monobasic potassium phosphate and 0.25 g/L of dibasic potassium phosphate

  Solution A: Acetonitrile and Buffer A (50:50)

  Solution B: Acetonitrile and Buffer A (65:35)

  Solution C: Butyl alcohol and Buffer A (8:92)

  Mobile phase: Acetonitrile, butyl alcohol, tetrahydrofuran (stabilizer-free), and Buffer B (18:5:8:69). Adjust with 1 M phosphoric acid or 1 M potassium hydroxide to a pH of 6.3 ± 0.1, if necessary.

  Cleaning solution: Acetonitrile, butyl alcohol, tetrahydrofuran (stabilizer-free), and Buffer A (30:8:13:49)

  **Peak identification solution:** Transfer 5–10 g of Oral Solution to a suitable sealed container. Add an amount of citric acid equivalent to 1% by weight of Oral Solution taken, and mix until dissolved. Seal the container, and heat...
at 70° for 24 h. Transfer 5.0 mL of the thermally degraded sample to a 200-mL volumetric flask. Dissolve and dilute with Solution B to volume.

**Standard stock solution:** 0.1 mg/mL of USP Ritonavir RS in Solution A

**Standard solution:** 10 µg/mL of USP Ritonavir RS in Solution C from the Standard stock solution

**Sample stock solution:** Nominally 2 mg/mL of ritonavir in Solution B prepared as follows. Transfer a measured volume of Oral Solution equivalent to 400 mg of ritonavir to a 200-mL volumetric flask, and dilute with Solution B to volume.

**Sample solution:** Nominally 1 mg/mL of ritonavir prepared as follows. Transfer 25.0 mL of the Sample stock solution to a 50-mL volumetric flask, and dilute with Solution C to volume. Transfer 15.0 mL of the solution to a 50-mL centrifuge tube that has been previously rinsed with methanol and dried. Add 20.0 mL of heptane, then stopper the tube. Shake the tube vigorously until a uniform emulsion is obtained, making sure to vent periodically. Centrifuge the resulting emulsion for about 5 min. Carefully aspirate off the top layer (heptane), leaving the clear bottom layer (Sample solution) in the tube. The centrifuged emulsion will have three distinct layers. The top layer (clear heptane) and the bottom layer (Sample solution) are separated by a viscous white cloudy layer. The middle layer should be considered part of the top layer for removal by aspiration. Repeat the extraction steps, and analyze the Sample solution.

**Chromatographic system**

(See Chromatography (621), System Suitability.)

**Mode:** LC

**Detector:** UV 240 nm

**Column:** 4.6-mm × 15-cm; 3-µm packing L26. Wash the column with the Cleaning solution after each injection of the Sample solution and each injection of the Standard solution for about 26 min, and equilibrate with Mobile phase for about 30 min. Store in the Cleaning solution after the analysis is completed.

**Column temperature:** 60°

**Flow rate:** 1 mL/min

**Injection volume:** 50 µL

**Run time:** 1.8 times the retention time of ritonavir

**System suitability**

**Sample:** Standard solution

**Suitability requirements**

**Tailing factor:** 0.8–1.2

**Relative standard deviation:** NMT 3.0%

**Analysis**

**Samples:** Peak identification solution, Standard solution, and Sample solution

[Note—Determine the relative retention value (r) for the components listed in Table 1 as directed in Chromatography (621), using the time measured at the baseline deflection of the Standard solution chromatogram as the void volume (t\text{Void}).]

Calculate the percentage of each impurity in the portion of Oral Solution taken:

\[
F = \text{relative response factor (see Table 1)}
\]

**Acceptance criteria:** See Table 1. Disregard peaks less than 0.05%.

**Table 1**

<table>
<thead>
<tr>
<th>Name</th>
<th>Relative Retention (r)</th>
<th>Relative Response Factor</th>
<th>Acceptance Criteria, NMT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ureidovaline(^h)</td>
<td>0.03</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>N-Deacetylvaline ritonavir(^c)</td>
<td>0.11</td>
<td>1.0</td>
<td>0.7</td>
</tr>
<tr>
<td>Acetamidoalcohol(^h)</td>
<td>0.15</td>
<td>1.0</td>
<td>0.1</td>
</tr>
<tr>
<td>Hydroxypropyl carbamate analog(^d)</td>
<td>0.59</td>
<td>0.4</td>
<td>—</td>
</tr>
<tr>
<td>2,5-Thiazolylmethylcarbamate(^h)</td>
<td>0.24</td>
<td>1.37</td>
<td>0.1</td>
</tr>
<tr>
<td>Hydroxyritonavir(^g)</td>
<td>0.36</td>
<td>1.0</td>
<td>0.5</td>
</tr>
<tr>
<td>Hydantoin aminocarboxylic analog(^h)</td>
<td>0.39</td>
<td>0.73</td>
<td>0.5</td>
</tr>
<tr>
<td>Ritonavir hydroxydicarbamate(^h)</td>
<td>0.45</td>
<td>0.66</td>
<td>1.0</td>
</tr>
<tr>
<td>Ethyl carbamate analog(^d)</td>
<td>0.44</td>
<td>1.0</td>
<td>0.2</td>
</tr>
<tr>
<td>Hydantoin-oxazolidinone derivative(^h)</td>
<td>0.50</td>
<td>0.76</td>
<td>0.2</td>
</tr>
<tr>
<td>Ethyl analog(^h)</td>
<td>0.64</td>
<td>1.0</td>
<td>0.1</td>
</tr>
<tr>
<td>O-Acyl isomer(^h)</td>
<td>0.74</td>
<td>1.0</td>
<td>0.2</td>
</tr>
<tr>
<td>BOC-aminocarboxyl [^h]</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Isobutoxycarbonyl aminocarboxyl [^h]</td>
<td>0.81</td>
<td>0.74</td>
<td>0.1</td>
</tr>
<tr>
<td>Oxazolidinone derivative(^h)</td>
<td>0.87</td>
<td>0.53</td>
<td>0.2</td>
</tr>
<tr>
<td>Ureidovaline isobutyl ester(^h)</td>
<td>0.94</td>
<td>1.0</td>
<td>0.1</td>
</tr>
<tr>
<td>Ritonavir</td>
<td>1.00</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>4-Hydroxy isomer(^h)</td>
<td>1.05</td>
<td>1.0</td>
<td>0.1</td>
</tr>
<tr>
<td>3R-Epimer(^h)</td>
<td>1.11</td>
<td>1.0</td>
<td>0.3</td>
</tr>
<tr>
<td>Aminoalcohol urea derivative(^h)</td>
<td>1.14</td>
<td>1.0</td>
<td>0.1</td>
</tr>
<tr>
<td>3R,5R-Diastereomer(^h)</td>
<td>1.23</td>
<td>1.0</td>
<td>0.1</td>
</tr>
<tr>
<td>5R-Epimer(^h)</td>
<td>1.32</td>
<td>1.0</td>
<td>0.1</td>
</tr>
<tr>
<td>Diacyl valine urea(^h)</td>
<td>1.70</td>
<td>1.0</td>
<td>0.1</td>
</tr>
<tr>
<td>Any individual unspecified degradation product</td>
<td>—</td>
<td>1.0</td>
<td>0.2</td>
</tr>
<tr>
<td>Total process impurity</td>
<td>—</td>
<td>—</td>
<td>1.2</td>
</tr>
</tbody>
</table>
Standard solution: 0.4% (v/v) of dehydrated alcohol prepared as follows. Transfer 10.0 mL of the Standard stock solution and 5 mL of the Internal standard solution to a 100-mL volumetric flask, and dilute with methanol to volume.

Sample stock solution: Transfer 5.0 mL of Oral Solution to a 50-mL volumetric flask with the aid of several portions of methanol, and dilute with methanol to volume.

Sample solution: Transfer 10.0 mL of the Sample stock solution and 5.0 mL of the Internal standard solution to a 100-mL volumetric flask, and dilute with methanol to volume.

**Chromatographic system**

(See Chromatography (621), System Suitability.)

**Mode:** GC

**Detector:** Flame ionization

**Column:** 0.53-mm × 30-m fused silica capillary; 1-μm layer of phase G16

**Temperatures**

** Injection port:** 185°C

** Detector:** 220°C

** Column:** See Table 2.

**Carrier gas:** Helium

**Flow rate:** 4.5 mL/min

**Makeup gas flow:** 30 mL/min

**Injection volume:** 1 μL

**Injection type:** Split injection with a split ratio, 4:1

**System suitability**

**Sample:** Standard solution

**Suitability requirements**

**Tailing factor:** 0.8–1.2 for the alcohol peak

**Relative standard deviation:** NMT 3.0% for the peak area ratio of alcohol to butyl alcohol

**Analysis**

**Samples:** Internal standard identity solution, Standard solution, and Sample solution

Calculate the percentage of alcohol in the portion of Oral Solution taken:

\[ \text{Result} = \left( \frac{R_a}{R_d} \right) \times C \times D \]

- **Acceptance criteria:** 40%–47% (v/v) of alcohol (C₆H₁₂O₁₁)

**Microbial Enumeration Tests** (61) and **Tests for Specified Microorganisms** (62): The total aerobic microbial count does not exceed 10² cfu/mL.

**ADDITIONAL REQUIREMENTS**

**Packaging and Storage:** Preserve in tight, light-resistant containers. Store at room temperature.
Rimantadine Hydrochloride

C₁₂H₂₁N·HCl 215.76
Tricyclo[3.3.1.1⁷]decane-1-methanamine, α-methyl-, hydrochloride.
α-Methyl-1-adamananemethylamine hydrochloride [1501-84-4].

» Rimantadine Hydrochloride contains not less than 98.0 percent and not more than 102.0 percent of C₁₂H₂₁N·HCl, calculated on the dried basis.

Packaging and storage—Preserve in well-closed containers, and store between 15° to 30°.

USP Reference standards (11)
USP Rimantadine Hydrochloride RS

Identification—

Change to read:

A: Spectroscopic Identification Tests (197), Infrared Spectroscopy: 197K
B: The retention time of the rimantadine peak in the chromatogram of the Assay preparation corresponds to that in the chromatogram of the Standard preparation, as obtained in the Assay.

X-ray diffraction (941)—The X-ray diffraction pattern conforms to that of USP Rimantadine Hydrochloride RS, similarly determined.

Loss on drying (731)—Dry it at 105° for 3 hours: it loses not more than 0.5% of its weight.

Residue on ignition (281): not more than 0.2%.

Ordinary impurities (466)—

Test solution—Transfer 100 mg of Rimantadine Hydrochloride to a 10-mL centrifuge tube, add 2 mL of 1 N sodium hydroxide, and mix. Add 2 mL of chloroform, and mix on a vortex mixer for 1 minute. Allow the layers to separate, and filter a portion of the top hexane layer into the 10-mL centrifuge tube, add 15 mL of 1 N sodium hydroxide, and mix. Add 25.0 mL of Internal standard solution, and shake by mechanical means for about 15 minutes. Allow the layers to separate, and filter a portion of the top hexane layer through anhydrous sodium sulfate. Use the clear filtrate.

Standard preparation—Proceed as directed for the Test solution, using USP Rimantadine Hydrochloride RS in place of the test specimen.

Eluant: a mixture of ethyl acetate, methanol, and ammonium hydroxide (80:10:4).

Procedure—Use a low-actinic glass tank. Dry the plate in a stream of hot air, then heat in an oven at 105° for 30 minutes. Allow the plate to cool to room temperature.

Visualization—Place the plate in an atmosphere of chlorine, prepared by mixing 1.5% potassium permanganate solution and diluted hydrochloric acid (1:1), for about 90 minutes. Allow to air-dry for 60 minutes, and follow with visualization technique 20.

Limit of toluene—

Standard solution—Transfer 10 µL of toluene to a 100-mL volumetric flask, dilute with chloroform to volume, and mix.

Test solution—Transfer about 750 mg of Rimantadine Hydrochloride, accurately weighed, to a 10-mL volumetric flask, dilute with chloroform to volume, and mix.

Chromatographic system (see Chromatography (621))—The gas chromatograph is equipped with a flame-ionization detector and a 2-mm × 2-m column that contains 80- to 100-mesh support S1A. The column temperature is maintained at about 200°, and nitrogen is used as the carrier gas. The injection port and detector temperatures are maintained at about 250°. Chromatograph the Standard solution, and record the peak responses as directed for Procedure: the tailing factor is not more than 1.5 for toluene; and the relative standard deviation for replicate injections is not more than 2.0%.

Procedure—Separately inject equal volumes (about 5 µL) of the Standard solution and the Test solution into the chromatograph, record the chromatograms for 9 minutes, and measure the responses for the toluene peaks. Calculate the percentage of toluene in the portion of Rimantadine Hydrochloride taken by the formula:

$$0.867(100/W_o)(r_o/r_s)$$

in which 0.867 is the specific gravity of toluene; W_o is the weight, in mg, of Rimantadine Hydrochloride taken to prepare the Test solution; and r_o and r_s are the toluene peak responses obtained from the Test solution and the Standard solution, respectively: not more than 0.1% is found.

Assay—

Internal standard solution—Transfer about 400 mg of n-eicosane to a 250-mL volumetric flask, dilute with hexane to volume, and mix.

Standard preparation—Transfer about 40 mg of USP Rimantadine Hydrochloride RS, accurately weighed, to a 50-mL centrifuge tube, add 15 mL of 1 N sodium hydroxide, and mix. Add 25.0 mL of Internal standard solution, and shake by mechanical means for about 15 minutes. Allow the layers to separate, and filter a portion of the top hexane layer through anhydrous sodium sulfate. Use the clear filtrate.

Assay preparation—Using about 40 mg of Rimantadine Hydrochloride, accurately weighed, proceed as directed for Standard preparation.

Chromatographic system (see Chromatography (621))—The gas chromatograph is equipped with a flame-ionization detector and a 4-mm × 1.8-m glass column that is packed with 3% phase G19 on 100- to 200-mesh support S1A. The column temperature is maintained at about 160°, and the injection port and detector temperatures are maintained at about 250°. Nitrogen is used as the carrier gas. Adjust the carrier flow rate and temperature so that the n-eicosane elutes at about 8 minutes. Chromatograph the Standard preparation, and record the peak responses as directed for Procedure: the tailing factor is not more than 2.0 for rimantadine; and the relative standard deviation for replicate injections is not more than 2.0%.

Procedure—Separately inject equal volumes (about 2 µL) of the Standard preparation and the Assay preparation into the chromatograph, record the chromatograms, and measure the responses for the major peaks. Calculate the quantity, in mg, of C₁₂H₂₁N·HCl in the portion of Rimantadine Hydrochloride taken by the formula:

$$25(C(r_o/r_s))$$

in which C is the concentration, in mg per mL, of USP Rimantadine Hydrochloride in the Standard preparation; and R_o and R_s are the ratios of the rimantadine peak response to the n-eicosane peak response obtained from the Assay preparation and the Standard preparation, respectively.
Rimantadine Hydrochloride Tablets

Rimantadine Hydrochloride Tablets contain not less than 90.0 percent and not more than 110.0 percent of the labeled amount of rimantadine hydrochloride (C\(_{12}\)H\(_{21}\)N·HCl).

Packaging and storage—Preserve in tight, light-resistant containers, and store between 15° to 30°.

USP Reference standards (11)—USP Rimantadine Hydrochloride RS

Identification—
A: The retention time of the rimantadine peak in the chromatogram of the Assay preparation corresponds to that in the chromatogram of the Standard preparation, as obtained in the Assay.

B: [CAUTION—Avoid contact with o-tolidine when performing this test, and conduct the test in a well-ventilated hood.] Weigh and finely powder not fewer than 5 Tablets. Transfer a portion of the powder, equivalent to 100 mg of rimantadine hydrochloride, to a 10-mL centrifuge tube, add 2 mL of 1 N sodium hydroxide, and mix. Add 2 mL of chloroform, and mix on a vortex mixer for 1 minute. Allow the layers to separate, and use the organic layer as the test solution. Separately apply 10 µL of the test solution and 10 µL of a Standard solution of USP Rimantadine Hydrochloride RS, similarly prepared, to a suitable thin-layer chromatographic plate (see Chromatography (621)) coated with a 0.25-mm layer of chromatographic silica gel. Place the plate in a low-actinic glass chromatographic chamber, and develop the chromatogram in a solvent system consisting of a mixture of ethyl acetate, methanol, and ammonium hydroxide (80:10:4) until the solvent front has moved about three-fourths of the length of the plate. Remove the plate from the chamber, dry it in a stream of hot air, and then heat in an oven at 105° for 30 minutes. Allow the plate to cool to room temperature. Place the dried plate in an atmosphere of chlorine, prepared from a mixture of 1.5% potassium permanganate solution and 3 N hydrochloric acid (1:1), for about 90 minutes. Remove the plate, and allow it to air-dry for 60 minutes. Prepare a spray reagent as follows. Dissolve 160 mg of o-tolidine in 30 mL of glacial acetic acid, dilute with water to 500 mL, add 1 g of potassium iodide, and mix until the potassium iodide is dissolved. Locate the spots on the plate by spraying with the spray reagent: the \( R_s \) value of the principal spot in the chromatogram of the test solution corresponds to that of the principal spot obtained from the Standard solution. Uniformity of dosage units (905): meet the requirements.

Dissolution (711)—
Medium: water; 900 mL.
Apparatus 2: 50 rpm.
Time: 30 minutes.

Procedure—Determine the amount of C\(_{12}\)H\(_{21}\)N·HCl dissolved, employing the procedure set forth in the Assay. Tolerances—Not less than 80% (Q) of the labeled amount of C\(_{12}\)H\(_{21}\)N·HCl is dissolved in 30 minutes.

Assay—
Internal standard solution, Standard preparation, and Chromatographic system—Proceed as directed in the Assay under Rimantadine Hydrochloride.

Assay preparation—Weigh and finely powder not fewer than 20 Tablets. Transfer an accurately weighed portion of the powder, equivalent to about 40 mg of rimantadine hydrochloride, to a 50-mL centrifuge tube, add 15 mL of 1 N sodium hydroxide, and mix. Add 25.0 mL of Internal standard solution, and shake by mechanical means for about 15 minutes. Allow the layers to separate, and filter a portion of the top hexane layer through anhydrous sodium sulfate. Use the clear filtrate as the Assay preparation.

Procedure—Separately inject equal volumes (about 2 µL) of the Standard preparation and the Assay preparation into the chromatograph, record the chromatograms, and measure the responses for the major peaks. Calculate the quantity, in mg, of rimantadine hydrochloride (C\(_{12}\)H\(_{21}\)N·HCl) in the portion of Tablets taken by the formula:

\[ 2SC(R_s/R_R) \]

Sodium Chloride Injection

DEFINITION
Sodium Chloride Injection is a sterile solution of Sodium Chloride in Water for Injection. It contains no antimicrobial agents. It contains NLT 95.0% and NMT 105.0% of the labeled amount of sodium chloride (NaCl).

IDENTIFICATION
• A. IDENTIFICATION TESTS—GENERAL, Sodium (191) and Chloride (191): Meets the requirements

ASSAY
• PROCEDURE
Sample solution: Transfer a volume of Injection, equivalent to 90 mg of sodium chloride, into a conical flask. Add water, if necessary, to bring the volume to 10 mL, and add 10 mL of glacial acetic acid, 75 mL of methanol, and 3 drops of eosin Y TS.

Analysis: Titrate, with shaking, with 0.1 N silver nitrate VS to a pink endpoint. Each mL of 0.1 N silver nitrate is equivalent to 5.844 mg of sodium chloride (NaCl).

Acceptance criteria: 95.0%–105.0%

IMPURITIES
• IRON (241)

Test preparation: Dilute 5.0 mL of Injection with water to 45 mL, and add 2 mL of hydrochloric acid.

Acceptance criteria: NMT 2 ppm

SPECIFIC TESTS
• PH (791): 4.5–7.0

• PARTICULATE MATTER IN INJECTIONS (788): Meets the requirements

• BACTERIAL ENDOTOXINS TEST (85): NMT 0.5 USP Endotoxin Unit/mL where the labeled amount of sodium chloride in the Injection is between 0.5% and 0.9%, and NMT 3.6 USP Endotoxin Units/mL where the labeled amount of sodium chloride in the Injection is between 3.0% and 24.3%

• OTHER REQUIREMENTS: It meets the requirements in Injections and Implanted Drug Products (1).

ADDITIONAL REQUIREMENTS
• PACKAGING AND STORAGE: Preserve in single-dose glass or plastic containers. Glass containers are preferably of Type I or Type II glass.

• LABELING: The label states the total osmolar concentration in mOsmol/L. Where the contents are less than 100 mL, or where the label states that the Injection is not for direct injection but is to be diluted before use, the label...
Sodium Pertechnetate Tc 99m Injection

Pertechnetic acid (H₉⁹mTcO₄⁻), sodium salt. Sodium pertechnetate (Na₉⁹mTcO₄⁻) [23288-60-0].

» Sodium Pertechnetate Tc 99m Injection is a sterile solution, suitable for intravenous or oral administration, containing radioactive technetium (⁹⁹ᵐTc) in the form of sodium pertechnetate and sufficient Sodium Chloride to make the solution isotonic. Technetium 99m is a radioactive nuclide formed by the radioactive decay of molybdenum 99. Molybdenum 99 is a radioactive isotope of molybdenum and may be formed by the neutron bombardment of molybdenum 98 or as a product of uranium fission.

Sodium Pertechnetate Tc 99m Injection contains not less than 90.0 percent and not more than 110.0 percent of the labeled amount of ⁹⁹ᵐTc at the date and hour stated on the label. Other chemical forms of ⁹⁹ᵐTc do not exceed 5 percent of the total radioactivity.

Packaging and storage—Preserve in single-dose or multiple-dose containers.

Labeling—If intended for intravenous use, label it with the information specified for Labeling (7), Labels and Labeling for Injectable Products. Label it also to include the following: the time and date of calibration; the amount of ⁹⁹ᵐTc as sodium pertechnetate expressed as total megabecquerels (millicuries) and as megabecquerels (millicuries) per mL on the date and at the time of calibration; a statement of the intended use, whether oral or intravenous; the expiration date; and the statement “Caution—Radioactive Material.” If the Injection has been prepared from molybdenum 99 produced from uranium fission, the label so states. The labeling indicates that in making dosage calculations, correction is to be made for radioactive decay, and also indicates that the radioactive half-life of ⁹⁹ᵐTc is 6.0 hours.

Radionuclide identification (see Radioactivity (821))—Its gamma-ray spectrum is identical to that of a specimen of ⁹⁹ᵐTc that exhibits a major photopeak having an energy of 0.140 MeV.

Bacterial Endotoxins Test (85)—The limit of endotoxin content is not more than 175/√V USP Endotoxin Unit per mL of the Injection, when compared with the USP Endotoxin R5, in which V is the maximum recommended total dose, in mL, at the expiration date or time.

pH (791): between 4.5 and 7.5.

Radiochemical purity—Place a volume of Injection, appropriately diluted, such that it provides a count rate of about 20,000 counts per minute, about 25 mm from one end of a 25- × 300-mm strip of chromatographic paper (see Chromatography (621)). Develop the chromatogram over a suitable period of time by ascending chromatography, using a mixture of acetone and 2 N hydrochloric acid (80:20). Allow the chromatogram to air-dry. Determine the radioactivity distribution by scanning the chromatogram with a suitable collimated radiation detector. The radioactivity of the pertechnetate band is not less than 95% of the total radioactivity in the test specimen. The R₂ value for the pertechnetate band (approximately 0.9) falls within ±10.0% of the value found for a known sodium pertechnetate Tc 99m specimen when determined under identical conditions.

Radionuclidic purity—Using a suitable counting assembly, determine the radioactivity of each radionuclidic impurity, in kBq per MBq (µCi per mCi) of technetium 99m, in the Injection by use of a calibrated system as directed under Radioactivity (821).

For Injection prepared from technetium 99m derived from parent molybdenum 99 formed as a result of neutron bombardment of stable molybdenum—

MOLYBDENUM 99—The presence of molybdenum 99 in the Injection is shown by its characteristic gamma-ray spectrum. The most prominent photopeak of this radioactive nuclide have energies of 0.181, 0.740, and 0.780 MeV. Molybdenum 99 decays with a radioactive half-life of 66.0 hours. The amount of molybdenum 99 is not greater than 0.15 kBq per MBq (0.15 µCi per mCi) of technetium 99m per administered dose in the Injection, at the time of administration.

OTHER GAMMA-EMITTING RADIONUCLIDIC IMPURITIES—The total amount of other gamma-emitting radionuclidic impurities does not exceed 0.5 kBq per MBq (0.5 µCi per mCi) of technetium 99m, and does not exceed 92 kBq (2.5 µCi) per administered dose of the Injection, at the time of administration.

For Injection prepared from technetium 99m derived from parent molybdenum 99 formed as a result of uranium fission—Gamma- and beta-emitting impurities—

MOLYBDENUM 99—The Injection meets the requirements set forth for the Injection prepared by neutron irradiation of stable molybdenum (see foregoing).

IODINE 131—The most prominent photopeak of this radioactive nuclide has an energy of 0.364 MeV. Iodine 131 decays with a radioactive half-life of 8.08 days. The concentration of iodine 131 is not more than 0.05 kBq per MBq (0.05 µCi per mCi) of technetium 99m, at the time of administration.

RUTHENIUM 103—The most prominent photopeak of this radioactive nuclide has an energy of 0.497 MeV. Ruthenium 103 decays with a radioactive half-life of 39.5 days. The concentration of ruthenium 103 is not more than 0.05 kBq per MBq (0.05 µCi per mCi) of technetium 99m, at the time of administration.

STRONTIUM 89—Determine the presence of strontium 89 in the Injection by a counting system appropriate for the detection of particulate radiations. Strontium 89 decays by a beta emission with a maximum energy of 1.463 MeV, and a radioactive half-life of 52.7 days. Strontium 89 may be present in a concentration of not more than 0.0006 kBq per MBq (0.0006 µCi per mCi) of technetium 99m, at the time of administration.

STRONTIUM 90—Determine the presence of strontium 90 in the Injection by a counting system appropriate for the detection of particulate radiations. Strontium 90 decays by a beta emission with a maximum energy of 0.546 MeV, and a radioactive half-life of 27.7 years. Strontium 90 may be present in a concentration of not more than 0.00006 kBq per MBq (0.00006 µCi per mCi) of technetium 99m, at the time of administration.

ALL OTHER RADIONUCLIDIC IMPURITIES—Not more than 0.01% of all other beta and gamma emitters is present at the time of administration. Not more than 0.001 Bq of gross alpha impurity per 1 MBq (or 0.001 nCi of gross alpha impurity per 1 mCi) of technetium 99m is present at the time of administration.
**Chemical purity**

Aluminum (To be determined if separation is accomplished by an alumina column in the preparation of the Injection)—

**ALUMINUM STANDARD SOLUTION**—Dissolve 35.17 mg, accurately weighed, of aluminum potassium sulfate dodecahydrate in water to make 1000.0 mL. Each mL of this solution contains 2 µg of Al.

**PROCEDURE**—Pipet 10 mL of Aluminum standard solution into each of two 50-mL volumetric flasks. To each flask add 3 drops of methyl orange TS and 2 drops of 6 N ammonium hydroxide, then add 0.5 N hydrochloric acid, dropwise, until the solution turns red. To one flask add 25 mL of sodium thioglycolate TS, and to the other flask add 1 mL of edetate disodium TS. To each flask add 5 mL of eriochrome cyanine TS and 5 mL of acetate buffer TS, and add water to volume. Immediately determine the absorbance of the solution containing sodium thioglycolate TS at the wavelength of maximum absorbance at about 535 nm, with a suitable spectrophotometer, using the solution containing the edetate disodium TS as a blank. Repeat the procedure using two 1.0-mL aliquots of Injection. Calculate the quantity, in µg per mL, of aluminum in the Injection taken by the formula:

\[
20 \left( \frac{T_u}{T_s} \right)
\]

in which \(T_u\) and \(T_s\) are the absorbances of the solution from the Injection and the solution containing the aluminum standard, respectively. The concentration of aluminum ion in the Injection is not greater than 10 µg per mL.

**Methyl ethyl ketone** (To be determined if separation is accomplished by liquid-liquid extraction in the preparation of the Injection)—Place 1.0 mL of the Injection in a suitable container, and dilute with water to 20.0 mL. Add 2.0 mL of 1 N sodium hydroxide mix, then add 2.0 mL of 0.1 N iodine, dropwise, and again mix. At the same time, prepare a standard by placing 1.0 mL of a solution of methyl ethyl ketone (1 in 1000) in a similar container and diluting with water to 20.0 mL. Add 2.0 mL of 1 N sodium hydroxide, mix, then add 2.0 mL of 0.1 N iodine, dropwise, and again mix. After 2 minutes, the turbidity of the test specimen does not exceed that of the standard (0.1%).

**Other requirements**—It meets the requirements under Injections and Implanted Drug Products (1), except that the Injection may be distributed or dispensed prior to the completion of the test for Sterility, the latter test being started on the day of manufacture, and except that it is not subject to the recommendation on Container Content.

**Assay for radioactivity** (See Radioactivity (821)—Using a suitable counting assembly, determine the radioactivity, in MBq (mCi) per mL, in Injection by use of a calibrated system.

[NOTE—Purified Water whether it is available in bulk or packaged forms, is intended for use as an ingredient of official preparations and in tests and assays unless otherwise specified (see General Notices, 8.230. Water). Where used for sterile dosage forms, other than for parenteral administration, process the article to meet the requirements under Sterility Tests (71), or first render the Purified Water sterile and thereafter protect it from microbial contamination. Do not use Purified Water in preparations intended for parenteral administration. For such purposes use Water for Injection, Bacteriostatic Water for Injection, or Sterile Water for Injection. In addition to the Specific Tests, Purified Water that is packaged for commercial use elsewhere meets the additional requirements for Packaging and Storage and Labeling as indicated under Additional Requirements.]

**SPECIFIC TESTS**

[NOTE—Required for bulk and packaged forms of Purified Water]

- **TOTAL ORGANIC CARBON (643);** Meets the requirements
- **WATER CONDUCTIVITY, Bulk Water (645);** Meets the requirements

**ADDITIONAL REQUIREMENTS**

[NOTE—Required for packaged forms of Purified Water]

- **PACKAGING AND STORAGE:** Where packaged, preserve in unreactive storage containers that are designed to prevent microbial entry.
- **LABELING:** Where packaged, label it to indicate the method of preparation and that it is not intended for parenteral administration.

**Delete the following:**

▲**USP REFERENCE STANDARDS** (11)

USP 1,4-Benzooquinone RS▲ (ERR 1-Nov-2018)

**Zanamivir**

![Zanamivir Structure](image)

\[
\text{C}_{13}\text{H}_{23}\text{N}_{2}\text{O}_{5} \quad 332.31
\]

D-glycero-D-galacto-Non-2-enonic acid, 5-(acetylamino)-4-

\[(\text{aminoiminomethyl})\text{amine}]\text{-2,6-anhydro-3,4,5-trideoxy-;}

5-Acetamido-2,6-anhydro-3,4,5-trIDEOxy-4-guanidino-D-

glycero-D-galacto-non-2-enonic acid [139110-80-8].

**DEFINITION**

Zanamivir contains NLT 98.0% and NMT 102.0% of zanamivir \((C_{13}H_{23}N_{2}O_{5})\), calculated on the anhydrous and solvent-free basis.

**IDENTIFICATION**

[Change to read:

- **A. SPECTROSCOPIC IDENTIFICATION TESTS** (197), Infrared Spectroscopy: 197K or 197M▲ (CN 1-May-2020)
  Wavenumber range: 4000 cm\(^{-1}\) to 400 cm\(^{-1}\)**]
• B. The retention time of the major peak of the Sample solution corresponds to that of the Standard solution, as obtained in the Assay.

ASSAY

• PROCEDURE

Mobile phase: Acetonitrile and 7.5 mM sulfuric acid (60:40). Adjust with ammonia TS to a pH of 6.2.

Resolution solution: Prepare 2.5 µg/mL of talo-zanamivir and 0.05 mg/mL of zanamivir from USP Zanamivir Resolution Mixture RS in Mobile phase.

Standard solution: 0.045 mg/mL of USP Zanamivir RS in Mobile phase

Sample solution: 0.045 mg/mL of Zanamivir in Mobile phase

Chromatographic system

(See Chromatography (621), System Suitability.)

Detector: UV 234 nm

Column: 4.6-mm × 25-cm; 5-µm packing L82

Column temperature: 30°

Flow rate: 1.5 mL/min

Injection volume: 20 µL

System suitability

Samples: Resolution solution and Standard solution

Suitability requirements

Resolution: NLT 1.5 between the peaks of talo-zanamivir and zanamivir at 234 nm

Acceptance criteria:

Relative standard deviation: NMT 1.5%, Standard solution

Analysis

Samples: Standard solution and Sample solution

Calculate the percentage of zanamivir (C$_{12}$H$_{28}$N$_{4}$O$_{5}$) in the portion of Zanamivir taken:

\[ \text{Result} = \left( \frac{r_i}{r_o} \right) \times \left( \frac{C_i}{C_o} \right) \times 100 \]

\( r_i \) = peak response from the Sample solution

\( r_o \) = peak response from the Standard solution

\( C_i \) = concentration of USP Zanamivir RS in the Standard solution (mg/mL)

\( C_o \) = concentration of Zanamivir in the Sample solution (mg/mL)

Acceptance criteria: 98.0%–102.0% on the anhydrous and solvent-free basis

IMPURITIES

• ORGANIC IMPURITIES

Mobile phase: Proceed as directed in the Assay.

System suitability solution: Prepare 0.45 mg/mL of zanamivir and 9 µg/mL each of imidazole, imidazole carboximidamide, zanamivir urea analog, 4-amino zanamivir, 4-biguanide zanamivir, and talo-zanamivir from USP Zanamivir Related Compounds Mixture RS in Mobile phase.

Sample solution: 0.45 mg/mL of Zanamivir prepared as follows. Dissolve the sample using 40% of the final volume with water, and dilute with acetonitrile to volume.

Chromatographic system

(See Chromatography (621), System Suitability.)

Mode: LC

Detector: UV 210 nm and 234 nm

Column: 4.6-mm × 25-cm; 5-µm packing L82

Column temperature: 30°

Flow rate: 1.5 mL/min

Injection volume: 20 µL

System suitability

Sample: System suitability solution

Suitability requirements

Resolution: NLT 1.5 between the peaks of talo-zanamivir and zanamivir at 234 nm

Analysis

Sample: Sample solution

Calculate the percentage of imidazole in the portion of Zanamivir taken:

\[ \text{Result} = \left( \frac{r_i}{r_o} \right) \times \left( \frac{F}{F + r_s} \right) \times 100 \]

\( r_i \) = peak response of imidazole carboximidamide or 4-biguanide zanamivir at 234 nm

\( F \) = relative response factor for imidazole (see Table 1)

\( r_s \) = peak response of zanamivir at 234 nm

Calculate the percentage of imidazole carboximidamide and 4-biguanide zanamivir in the portion of Zanamivir taken:

\[ \text{Result} = \left( \frac{r_i}{r_o} \right) \times \left( \frac{F}{F + r_s} \right) \times 100 \]

\( r_i \) = peak response of imidazole carboximidamide or 4-biguanide zanamivir at 234 nm

\( F \) = relative response factor for imidazole carboximidamide or 4-biguanide zanamivir (see Table 1)

\( r_s \) = sum of the responses of all the peaks including the zanamivir peak at 234 nm

Calculate the percentage of O-triazinyl zanamivir, zanamivir urea analog, 4-amino zanamivir, talo-zanamivir, zanamivir dimer, and any other unspecified impurity in the portion of Zanamivir taken:

\[ \text{Result} = \left( \frac{r_i}{r_o} \right) \times 100 \]

\( r_i \) = peak response of O-triazinyl zanamivir, zanamivir urea analog, 4-amino zanamivir, talo-zanamivir, zanamivir dimer, or any other unspecified impurity at 234 nm

\( r_o \) = sum of the responses of all the peaks including the zanamivir peak at 234 nm

Acceptance criteria: See Table 1.

<table>
<thead>
<tr>
<th>Name</th>
<th>Relative Retention Time</th>
<th>Relative Response Factor</th>
<th>Acceptance Criteria, NMT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imidazole*</td>
<td>0.26</td>
<td>2.5</td>
<td>—</td>
</tr>
<tr>
<td>Imidazole carboximidamide*</td>
<td>0.30</td>
<td>3.3</td>
<td>0.01</td>
</tr>
<tr>
<td>O-Triazinyl zanamivir*</td>
<td>0.60</td>
<td>—</td>
<td>0.3</td>
</tr>
<tr>
<td>Zanamivir urea analog*</td>
<td>0.70</td>
<td>—</td>
<td>0.2</td>
</tr>
<tr>
<td>4-Amino zanamivir*</td>
<td>0.77</td>
<td>—</td>
<td>0.2</td>
</tr>
<tr>
<td>4-Biguanide zanamivir*</td>
<td>0.83</td>
<td>1.6</td>
<td>0.2</td>
</tr>
<tr>
<td>Zanamivir</td>
<td>1.00</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>talo-Zanamivir*</td>
<td>1.14</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Zanamivir dimer*</td>
<td>2.75</td>
<td>—</td>
<td>0.5</td>
</tr>
<tr>
<td>Any other unspecified impurity</td>
<td>—</td>
<td>—</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Published on March 26, 2020
### Table 1 (continued)

<table>
<thead>
<tr>
<th>Name</th>
<th>Relative Retention Time</th>
<th>Relative Response Factor</th>
<th>Acceptance Criteria, NMT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total impurities</td>
<td>—</td>
<td>—</td>
<td>1.2</td>
</tr>
</tbody>
</table>

---

*1H-Imidazole (No individual limit. Included in the determination of total impurities.)*

*1H-Imidazole-1-carboximidamide.*

*5-Acetamido-9-O-[4-amino-6-(1H-pyrazol-1-yl)-1,3,5-triazin-2-yl]-2,6-anhydro-3,4,5-trideoxy-4-guanidino-D-glycero-D-galacto-non-2-enonic acid.*

*5-Acetamido-9-O-[4-amino-6-(1H-pyrazol-1-yl)-1,3,5-triazin-2-yl]-2,6-anhydro-3,4,5-trideoxy-4-ureido-D-glycero-D-galacto-non-2-enonic acid.*

*5-Acetamido-9-O-[4-amino-6-(1H-pyrazol-1-yl)-1,3,5-triazin-2-yl]-2,6-anhydro-3,4,5-trideoxy-4-amino-D-glycero-D-galacto-non-2-enonic acid.*

*5-Acetamido-2,6-anhydro-3,4,5-trideoxy-4-guanidino-D-glycero-D-talo-non-2-enonic acid (No individual limit. Included in the determination of total impurities.)*

*4,4'-[(2-Amino-4-oxo-1,3,5-triazapent-2-ene-1,5-diyl) bis(5-acetamido-2,6-anhydro-3,4,5-trideoxy-D-glycero-D-galacto-non-2-enonic acid.)*

**SPECIFIC TESTS**

**Optical Rotation, Specific Rotation (7815)**

Sample solution: 10 mg/mL in water

Acceptance criteria: +36.0° to +38.0°, measured at 20°C, determined on the anhydrous and solvent-free basis

**Water Determination, Method Ic (921):** 4.0%–9.0%

**ADDITIONAL REQUIREMENTS**

**Packaging and Storage:** Store in a well-closed container at controlled room temperature.

---

**USP Reference Standards**

**USP Zanamivir RS**

**USP Zanamivir Related Compounds Mixture RS**

This Reference Standard is a mixture of zanamivir, imidazole, imidazole carboximidamide, zanamivir urea analog, 4-amino zanamivir, 4-biguanide zanamivir, talo-zanamivir, and zanamivir dimer. The chemical names are given below:

*Imidazole: 1H-Imidazole.*

*Imidazole carboximidamide: 1H-Imidazole-1-carboximidamide.*

*Zanamivir urea analog: 5-Acetamido-9-O-[4-amino-6-(1H-pyrazol-1-yl)-1,3,5-triazin-2-yl]-2,6-anhydro-3,4,5-trideoxy-4-ureido-D-glycero-D-galacto-non-2-enonic acid.*

*4-Amino zanamivir: 5-Acetamido-9-O-[4-amino-6-(1H-pyrazol-1-yl)-1,3,5-triazin-2-yl]-2,6-anhydro-3,4,5-trideoxy-4-amino-D-glycero-D-galacto-non-2-enonic acid.*

*4-Biguanide zanamivir: 5-Acetamido-9-O-[4-amino-6-(1H-pyrazol-1-yl)-1,3,5-triazin-2-yl]-2,6-anhydro-3,4,5-trideoxy-4-(1-biguanidyl)-D-glycero-D-galacto-non-2-enonic acid.*

*talo-Zanamivir: 5-Acetamido-2,6-anhydro-3,4,5-trideoxy-4-guanidino-D-glycero-D-talo-non-2-enonic acid.*

USP Zanamivir Resolution Mixture RS

This Reference Standard is a mixture of zanamivir and talo-zanamivir.
Excipients

USP and NF Excipients, Listed by Functional Category

In the following reference table, the grouping of excipients by functional category is intended to summarize commonly identified purposes that these excipients serve in drug product formulations. The association of a functional category with a particular dosage form in this table is not absolute and does not limit the use of an excipient to a single type of dosage form or delivery system.

**Adhesive**

**Dosage Form:** Transdermals and “Patches”
- Dimethicone
- Polyisobutylene

**Air Displacement**

- Carbon Dioxide
- Nitrogen

**Alcohol Denaturant**

- Denatonium Benzoate
- Methyl Isobutyl Ketone
- Sucrose Octaacetate

**Antifoaming Agent**

- Dimethicone
- Lauric Acid
- Myristic Acid
- Palmitic Acid
- Simethicone

**Antimicrobial Preservative**

**Dosage Form:** Oral Liquids
- Alcohol
- Benzalkonium Chloride
- Benzalkonium Chloride Solution
- Benzenethionium Chloride
- Benzoic Acid
- Benzyl Alcohol
- Boric Acid
- Butylparaben
- Calcium Acetate
- Calcium Chloride
- Calcium Lactate
- Calcium Propionate
- Cetrimonium Bromide
- Cetylpyridinium Chloride
- Chlorobutanol
- Chlorocresol
- Chloroxylenol
- Cresol
- Dehydroacetic Acid
- Erythorbic Acid
- Ethylparaben
- Ethylparaben Sodium
- Glycerin
- Imidurea
- Mandelic Acid
- Methylparaben
- Methylparaben Sodium
- Monothioglycerol
- Pentetic Acid
- Phenol
- Phenoxycethanol
- Phenylethyl Alcohol
- Phenylmercuric Acetate
- Phenylmercuric Nitrate
- Potassium Benzoate
- Potassium Metabisulfite
- Potassium Sorbate
- Propionic Acid
- Propylene Glycol
- Propylparaben
- Propylparaben Sodium
- Sodium Acetate
- Sodium Benzoate
- Sodium Borate
- Sodium Dehydroacetate
- Sodium Lactate Solution
- Sodium Metabisulfite
- Sodium Propionate
- Sodium Sulfite
- Sorbic Acid
- Sulfur Dioxide
- Thimerosal
- Thymol
- Zinc Oxide

**Dosage Form:** Ophthalmic Preparations
- Benzalkonium Chloride

Published on March 26, 2020
Benzyl Alcohol
Chlorobutanol
Propylparaben
Sorbic Acid

**Antioxidant**

**Dosage Form:** Oral Liquids
- Ascorbic Acid
- Ascorbyl Palmitate
- Butylated Hydroxyanisole
- Butylated Hydroxytoluene
- Citric Acid Monohydrate
- Erythorobic Acid
- Fumaric Acid
- Hypophosphorous Acid
- Lactobionic Acid
- Malic Acid
- Methionine
- Monothioglycerol
- Potassium Metabisulfite
- Propionic Acid
- Propyl Gallate
- Racemethionine
- Sodium Ascorbate
- Sodium Bisulfite
- Sodium Formaldehyde Sulfoxylate
- Sodium Metabisulfite
- Sodium Sulfite
- Sodium Thiosulfate
- Stannous Chloride
- Sulfur Dioxide
- Thymol
- Tocopherol
- Tocopherols Excipient
- Vitamin E
- Vitamin E Polyethylene Glycol Succinate

**Bulking Agent**
- Alpha-Lactalbumin
- Polydextrose
- Polydextrose, Hydrogenated
- Pullulan

**Dosage Form:** Parenterals
- Creatinine
- Glycine
- Mannitol
- Trehalose

**Capsule Shell**

**Dosage Form:** Tablets and Capsules
- Gelatin
- Hypromellose
- Pullulan

**Dosage Form:** Dry Powder Inhalers
- Gelatin
- Hypromellose

**Carrier**

**Dosage Form:** Dry Powder Inhalers
- Lactose, Anhydrous
- Lactose, Monohydrate

**Chelating and/or Complexing Agent**

**Dosage Form:** Oral Liquids
- Alfadex
- Betadex
- Betadex Sulfbutyl Ether Sodium
- Citric Acid Monohydrate
- Edetate Calcium Disodium
- Edetate Disodium
- Edetic Acid
- Galactose
- Gamma Cyclodextrin
- Hydroxpropyl Betadex
- Alpha-Lactalbomin
- Malic Acid
- Oxyquinoline Sulfate
- Pentetic Acid
- Potassium Citrate
- Sodium Phosphate, Dibasic
- Sodium Phosphate, Monobasic

**Change to read:**

**Coating Agent**

**Dosage Form:** Tablets and Capsules
- Amino Methacrylate Copolymer
- Ammonio Methacrylate Copolymer
- Ammonio Methacrylate Copolymer Dispersion
- Calcium Carbonate
- Carboxymethylcellulose Calcium
- Carboxymethylcellulose Sodium
- Carboxymethylcellulose Sodium, Enzymatically-Hydrolyzed
- Cellaburate
- Cellacefate
- Cellulose Acetate
- Cetyl Alcohol
- Chitosan
- Coconut Oil
- Coconut Oil, Hydrogenated
- Copovidone
- Corn Syrup Solids
- Ethyl Acrylate and Methyl Methacrylate Copolymer Dispersion
- Ethylcellulose
- Ethylcellulose Aqueous Dispersion
- Ethylcellulose Dispersion Type B
- Ethylene Glycol and Vinyl Alcohol Graft Copolymer Gelatin
- Glaze, Pharmaceutical
- Glucose, Liquid
- Glyceryl Behenate

▲ ▲ (NF 1-Aug-2020) ▲ ▲ (NF 1-Aug-2020)

- Glyceryl Dibehenate
- Hydroxyethyl Cellulose
Hydroxypropyl Cellulose
Hypermellose
Hypermellose Acetate Succinate
Hypermellose Phthalate
Isomalt
Alpha-Lactalbumin
Maltitol
Maltodextrin
Methacrylic Acid and Ethyl Acrylate Copolymer
Methacrylic Acid and Ethyl Acrylate Copolymer Dispersion
Methacrylic Acid and Ethyl Acrylate Copolymer, Partially-Neutralized
Methacrylic Acid and Methyl Methacrylate Copolymer
Methylcellulose
Palm Kernel Oil
Palm Oil
Palm Oil, Hydrogenated
Polydextrose
Polydextrose, Hydrogenated
Polyethylene Glycol
Polyethylene Glycol 3350
Polyethylene Oxide
Polyvinyl Acetate
Polyvinyl Acetate Dispersion
Polyvinyl Acetate Phthalate
Polyvinyl Alcohol
Pullulan
Rapeseed Oil, Fully Hydrogenated
Rapeseed Oil, Superglycerinated Fully Hydrogenated
Shellac
Starch, Pregelatinized Modified
Sucrose
Sugar, Confectioner’s
Sunflower Oil
Titanium Dioxide
Wax, Carnauba
Wax, Microcrystalline
Xylitol
Zein
Zinc Oxide

**Colloid Stabilizing Agent**

*Dosage Form:* Radiopharmaceuticals

Gelatin

**Coloring Agent**

Caramel
Ferric Oxide
Ferrosol Ferric Oxide

*Dosage Form:* Tablets and Capsules

Aluminum Oxide

**Desiccant**

Calcium Chloride
Calcium Sulfate
Polyvinyl Acetate
Silicon Dioxide

---

**Change to read:**

**Diluent**

▲Sucrose Diacetate Hexaisobutyrate▲ (NF 1-Aug-2020)

*Dosage Form:* Tablets and Capsules

Amino Methacrylate Copolymer
Ammonio Methacrylate Copolymer
Ammonio Methacrylate Copolymer Dispersion
Calcium Carbonate
Calcium Phosphate, Dibasic, Anhydrous
Calcium Phosphate, Dibasic, Dihydrate
Calcium Phosphate, Tribasic
Calcium Sulfate
Cellaburate
Cellulose, Microcrystalline
Cellulose, Silicified Microcrystalline
Cellulose, Powdered
Cellulose Acetate
Corn Syrup
Corn Syrup Solids
Dextrates
Dextrin
Dextrose
Dextrose Excipient
Erythritol
Ethyl Acrylate and Methyl Methacrylate Copolymer Dispersion
Fructose
Invert Sugar
Isomalt
Kaolin
Alpha-Lactalbumin
Lactitol
Lactose, Anhydrous
Lactose, Monohydrate
Magnesium Carbonate
Magnesium Oxide
Maltitol
Maltodextrin
Maltose
Mannitol
Methacrylic Acid and Ethyl AcrylateCopolymer
Methacrylic Acid and Ethyl Acrylate Copolymer Dispersion
Methacrylic Acid and Methyl Methacrylate Copolymer
Polydextrose
Polyethylene Glycol
Polyethylene Glycol 3350
Propylene Glycol Monocaprylate
Pullulan
Simethicone
Sodium Chloride
Sorbitol
Starch, Pregelatinized
Starch, Pregelatinized Modified
Starch, Corn
Starch, Hydroxypropyl Corn
Starch, Pregelatinized Hydroxypropyl Corn
Starch, Pea
Starch, Hydroxypropyl Pea

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Starch, Pregelatinized Hydroxypropyl Pea
Starch, Potato
Starch, Hydroxypropyl Potato
Starch, Pregelatinized Hydroxypropyl Potato
Starch, Tapioca
Starch, Wheat
Starch Hydrolysate, Hydrogenated
Sucrose
Sugar, Compressible
Sugar, Confectioner’s
Sugar Spheres
Sunflower Oil
Talc
Trehalose
Xylitol

Disintegrant

Dosage Form: Tablets and Capsules
Alginic Acid
Carboxymethylcellulose Calcium
Carboxymethylcellulose Sodium
Cellulose, Microcrystalline
Cellulose, Silicified Microcrystalline
Cellulose, Powdered
Crosclarmellose Sodium
Crospovidone
Glycine
Guar Gum
Hydroxypropyl Cellulose, Low-Substituted
Magnesium Aluminum Silicate
Maltose
Methycellulose
Polacrilin Potassium
Pullulan
Silicon Dioxide, Colloidal
Sodium Alginate
Sodium Starch Glycolate
Starch, Pregelatinized Modified
Starch, Corn
Starch, Hydroxypropyl Corn
Starch, Pregelatinized Hydroxypropyl Corn
Starch, Pea
Starch, Hydroxypropyl Pea
Starch, Pregelatinized Hydroxypropyl Pea
Starch, Potato
Starch, Hydroxypropyl Potato
Starch, Pregelatinized Hydroxypropyl Potato
Starch, Tapioca
Starch, Wheat
Trehalose

Emollient

Dosage Form: Semisolids, Topicals, and Suppositories
Alkyl (C12-15) Benzoate
Almond Oil
Aluminum Monostearate
Canola Oil
Castor Oil
Cetostearyl Alcohol
Cholesterol

Coconut Oil
Cyclomethicone
Dimethicone
Ethylene Glycol Stearates
Glycerin
Glycerol Monooleate
Glycerol Monostearate
Isopropyl Isostearate
Isopropyl Myristate
Isopropyl Palmitate
Isostearyl Isostearate
Hydrogenated Lanolin
Lecithin
Mineral Oil
Mineral Oil, Light
Myristyl Alcohol
Octyldecane
Oleyl Alcohol
Oleyl Oleate
Petrolatum
Polydecene, Hydrogenated
Polypropylene Glycol 11 Stearyl Ether
Propylene Glycol Dilaurate
Propylene Glycol Monolaurate
Safflower Oil
Soybean Oil, Hydrogenated
Sunflower Oil
Wax, Cetyl Esters
Xylitol
Zinc Acetate

Emulsifying Agent

Dosage Form: Oral Liquids
Acacia
Agar
Behenoyl Polyoxyglycerides
Benzalkonium Chloride
Benzy1 Benzoate
Caprylic Acid
Caprylocaproyl Polyoxyglycerides
Carbomer Copolymer
Carbomer Homopolymer
Carbomer Interpolymer
Carboxymethylcellulose Calcium
Cetostearyl Alcohol
Cetyl Alcohol
Cetylpyridinium Chloride
Cholesterol
Coconut Oil
Deoxocholic Acid

Desoxocholic Acid

[(Title for this monograph—not to change until December 1, 2021.) (Prior to December 1, 2021, the current practice of labeling the article of commerce with the name Deoxocholic Acid may be continued. Use of the name Deoxycholic Acid will be permitted as of December 1, 2016; however, the use of this name will not be mandatory until December 1, 2021. The 60-month extension will provide the time needed by manufacturers and users to make necessary changes.)]

Deoxocholic Acid
<table>
<thead>
<tr>
<th>Adjunct Name</th>
<th>Ingredient Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diethanolamine (Adjunct)</td>
<td>Propylene Glycol Alginate</td>
</tr>
<tr>
<td>Diethylene Glycol Monoethyl Ether</td>
<td>Propylene Glycol Diacetate</td>
</tr>
<tr>
<td>Diethylene Glycol Stearates</td>
<td>Propylene Glycol Dicaprylate/Dicaprate</td>
</tr>
<tr>
<td>Egg Phospholipids</td>
<td>Propylene Glycol Dilaurate</td>
</tr>
<tr>
<td>Ethylene Glycol Stearates</td>
<td>Propylene Glycol Monocaprylate</td>
</tr>
<tr>
<td>Glyceryl Distearate</td>
<td>Propylene Glycol Monolaurate</td>
</tr>
<tr>
<td>Glyceryl Mono and Dicaprylate</td>
<td>Propylene Glycol Monostearate</td>
</tr>
<tr>
<td>Glyceryl Monocaprylate</td>
<td>Rapeseed Oil, Superglycerinated Fully Hydrogenated</td>
</tr>
<tr>
<td>Glyceryl Monocaprylocaprate</td>
<td>Sodium Borate</td>
</tr>
<tr>
<td>Glyceryl Monolinoleate</td>
<td>Sodium Cetostearyl Sulfate</td>
</tr>
<tr>
<td>Glyceryl Monooleate</td>
<td>Sodium Lauryl Sulfate</td>
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<td>Isostearyl Isostearate</td>
<td>Sorbitan Trioleate</td>
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<tr>
<td>Alpha-Lactalbumin</td>
<td>Stannous Chloride</td>
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<tr>
<td>Lanolin</td>
<td>Starch, Hydroxypropyl Corn</td>
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<tr>
<td>Hydrogenated Lanolin</td>
<td>Starch, Hydroxypropyl Pea</td>
</tr>
<tr>
<td>Lanolin Alcohols</td>
<td>Starch, Hydroxypropyl Potato</td>
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<tr>
<td>Lauric Acid</td>
<td>Stearic Acid</td>
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<td>Lauroyl Polyoxylglycerides</td>
<td>Stearyl Polyoxylglycerides</td>
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<td>Lecithin</td>
<td>Sucrose Palmitate</td>
</tr>
<tr>
<td>Linoleoyl Polyoxylglycerides</td>
<td>Sucrose Stearate</td>
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<td>Magnesium Oxide</td>
<td>Sunflower Oil</td>
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<td>Medium-chain Triglycerides</td>
<td>Trolamine</td>
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<td>Methylcellulose</td>
<td>Vitamin E Polyethylene Glycol Succinate</td>
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<td>Mono- and Di-glycerides</td>
<td>Wax, Emulsifying</td>
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<td>Monoethanolamine (Adjunct)</td>
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<td>Octyldecanol</td>
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<td>Oleic Acid (Adjunct)</td>
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<td>Palm Kernel Oil</td>
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<td>Polyoxyl 15 Hydroxyoctaerste</td>
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<td>Polyoxyl 20 Cetostearyl Ether</td>
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<td>Polyoxyl 35 Castor Oil</td>
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<td>Polyoxyl 40 Castor Oil, Hydrogenated</td>
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<td>Polyoxyl 40 Stearate</td>
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<td>Polyoxyl Lauryl Ether</td>
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<td>Polyoxyl Stearyl Ether</td>
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<td>Polysorbate 80</td>
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<tr>
<td>Potassium Alginate</td>
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</table>

**Film-Forming Agent**

**Dosage Form:** Tablets and Capsules

- Alginic Acid
- Amino Methacrylate Copolymer
- Ammonio Methacrylate Copolymer
- Ammonio Methacrylate Copolymer Dispersion
- Carboxymethylcellulose Calcium
- Carboxymethylcellulose Sodium
- Carboxymethylcellulose Sodium, Enzymatically-Hydrolyzed
- Cellulose
- Cellulose Acetate
- Chitosan
- Copovidone
- Dibutyl Phthalate
- Diethyl Phthalate
- Ethyl Acrylate and Methyl Methacrylate Copolymer Dispersion
- Ethylcellulose
- Ethylcellulose Aqueous Dispersion
- Ethylcellulose Dispersion Type B
- Ethylene Glycol and Vinyl Alcohol Grafted Copolymer
- Gelatin
- Glaze, Pharmaceutical
- Hydroxyethyl Cellulose
- Hydroxypropyl Cellulose
- Hypromellose
- Hypromellose Acetate Succinate

Published on March 26, 2020
Hypromellose Phthalate
Methacrylic Acid and Ethyl Acrylate Copolymer
Methacrylic Acid and Ethyl Acrylate Copolymer, Partially-Neutralized
Methacrylic Acid and Methyl Methacrylate Copolymer
Methylcellulose
Polyethylene Glycol 3350
Polyvinyl Acetate Dispersion
Polyvinyl Acetate Phthalate
Polyvinyl Alcohol
Pullulan
Pyroxylin
Shellac
Sodium Alginate
Dosage Form: Transdermals and “Patches”
Chitosan
Dextrin
Gelatin
Hydroxyethyl Cellulose
Hypromellose
Pectin
Polyethylene Glycol
Polyvinyl Alcohol
Pullulan
Sodium Alginate
Xanthan Gum

Free Radical Scavenger
Dosage Form: Radiopharmaceuticals
Aminobenzoic Acid
Methylene Blue

Glidant and/or Anticaking Agent
Dosage Form: Tablets and Capsules
Calcium Phosphate, Tribasic
Calcium Silicate
Cellulose, Powdered
Magnesium Oxide
Magnesium Silicate
Magnesium Trisilicate
Silica, Dental-Type
Silica, Hydrophobic Colloidal
Silicon Dioxide, Colloidal
Sodium Stearate
Talc

Humectant
Corn Syrup Solids
Cyclomethicone
Erythritol
Glycerin
Hexylene Glycol
Inositol
Hydrogenated Lanolin
Maltitol
Polydextrose
Polydextrose, Hydrogenated
Propylene Glycol
Sodium Lactate Solution
Sorbitol
Sorbitol Sorbitan Solution
Starch Hydrolysate, Hydrogenated
Tagatose
Triacetin
Xylitol

Filtering Aid
Cellulose, Powdered
Siliceous Earth, Purified

Flavors and Fragrance
Anise Oil
Eucalyptus Oil
Isobutyl Alcohol
Neohesperidin Dihydrochalcone
Sodium Succinate
Star Anise Oil
Dosage Form: Tablets and Capsules
Adipic Acid
Almond Oil
Anethole
Benzyaldehyde
Denatonium Benzoate
Ethyl Acetate
Ethyl Maltol
Ethyl Vanillin
Ethylcellulose
Fructose
Fumaric Acid
L-Glutamic Acid, Hydrochloride
Lactitol
Leucine
Malic Acid
Maltol
Menthol
Methionine
Methyl Salicylate
Monosodium Glutamate
Peppermint
Peppermint Oil
Peppermint Spirit
Racemethionine
Rose Oil
Rose Water, Stronger
Sodium Acetate
Sodium Lactate Solution
Tartaric Acid
Thymol
Vanillin

Change to read:

Lubricant
Dosage Form: Tablets and Capsules
Behenoyl Polyoxyglycerides
Calcium Stearate
Castor Oil, Hydrogenated
Coconut Oil, Hydrogenated
Glyceryl Behenate ▲ ▲ (NF 1-Aug-2020) ▲ ▲ (NF 1-Aug-2020)
Glyceryl Dibehenate
Glyceryl Mono and Dicaprylate
Glyceryl Monocaprylate
Glyceryl Monocaprylocaprate
Glyceryl Tricaprylate
Glyceryl Tristearate
Lauric Acid
Magnesium Stearate
Mineral Oil, Light
Myristic Acid
Palm Oil, Hydrogenated
Palmitic Acid
Poloxamer
Polyethylene Glycol
Polyethylene Glycol 3350
Polyoxyl 10 Oleyl Ether
Polyoxyl 15 Hydroxystearate
Polyoxyl 20 Cetostearyl Ether
Polyoxyl 35 Castor Oil
Polyoxyl 40 Castor Oil, Hydrogenated
Polyoxyl 40 Stearate
Polysorbate 20
Polysorbate 40
Polysorbate 60
Polysorbate 80
Potassium Benzoate
Sodium Benzoate
Sodium Lauryl Sulfate
Sodium Stearate
Sodium Stearyl Fumarate
Sorbitan Monolaurate
Sorbitan Monostearate
Stearic Acid
Stearic Acid, Purified
Sucrose Stearate
Talc
Vegetable Oil, Hydrogenated, Type I
Zinc Stearate
Linoleyl Polyoxylglycerides
Ointment, Hydrophilic
Ointment, White
Ointment, Yellow
Oleoyl Polyoxylglycerides
Paraffin
Petrolatum
Petrolatum, Hydrophilic
Petrolatum, White
Polydecene, Hydrogenated
Polyethylene Glycol
Polyethylene Glycol 3350
Polyethylene Glycol Monomethyl Ether
Polyglyceryl 3 Diisostearate
Rose Water Ointment
Squalane
Stearoyl Polyoxylglycerides
Sucrose Diacetate Hexaisobutyrate ▲ (NF 1-Aug-2020)
Vegetable Oil, Hydrogenated, Type II
Vitamin E Polyethylene Glycol Succinate

**Pharmaceutical Water**

**Dosage Form:** Parenterals
Water for Injection
Water for Injection, Bacteriostatic
Water for Injection, Sterile
Water for Irrigation, Sterile
Water Purified
Water Purified, Sterile

**pH Modifier (Acidifying Agent/Alkalizing Agent/Buffering Agent)**
Sodium Succinate

**Dosage Form:** Oral Liquids
Acetic Acid
Acetic Acid, Glacial
Adipic Acid
Ammonia Solution, Strong
Ammonium Carbonate
Ammonium Chloride
Ammonium Phosphate
Boric Acid
Calcium Carbonate
Calcium Hydroxide
Calcium Lactate
Calcium Phosphate, Tribasic
Citric Acid Monohydrate
Citric Acid, Anhydrous
Diethanolamine
Fumaric Acid
Glycine
Hydrochloric Acid
Hydrochloric Acid, Diluted
Alpha-Lactalbuminum
Lactic Acid
Lysine Hydrochloride
Maleic Acid
Malic Acid
Methionine

---

**Ointment Base**

**Dosage Form:** Semisolids, Topicals, and Suppositories
Caprylocapryl Polyoxylglycerides
Coconut Oil
Diethylene Glycol Monoethyl Ether
Lanolin
Hydrogenated Lanolin
Lanolin Alcohols
Lauroyl Polyoxylglycerides

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Published on March 26, 2020
Monoethanolamine
Monosodium Glutamate
Nitric Acid
Phosphoric Acid
Phosphoric Acid, Diluted
Potassium Bicarbonate
Potassium Citrate
Potassium Hydroxide
Potassium Metaphosphate
Potassium Phosphate, Dibasic
Potassium Phosphate, Monobasic
Propionic Acid
Racemethionine
Sodium Acetate
Sodium Bicarbonate
Sodium Borate
Sodium Carbonate
Sodium Citrate
Sodium Hydroxide
Sodium Lactate Solution
Sodium Phosphate, Dibasic
Sodium Phosphate, Monobasic
Succinic Acid
Sulfuric Acid
Tartaric Acid
Trolamine

Change to read:

**Plasticizer**

**Dosage Form:** Tablets and Capsules
- Acetyltributyl Citrate
- Acetyltriethyl Citrate
- Benzyl Benzoate
- Castor Oil
- Chlorobutanol
- Diacetylated Monoglycerides
- Dibutyl Sebacate
- Diethyl Phthalate
- Glycerin
- Mannitol
- Polyethylene Glycol
- Polyethylene Glycol 3350
- Polyethylene Glycol Monomethyl Ether
- Propylene Glycol
- Pullulan
- Sorbitol
- Sorbitol Sorbitan Solution
- \(^\text{▲Sucrose Diacetae Hexaisobutyrate}^\text{▲ (NF 1-Aug-2020)}\)
- Triacetin
- Tributyl Citrate
- Triethyl Citrate
- Vitamin E

**Polymer Membrane**

**Dosage Form:** Tablets and Capsules
- Amino Methacrylate Copolymer
- Ammonio Methacrylate Copolymer
- Ammonio Methacrylate Copolymer Dispersion
- Cellaburate
- Cellulose Acetate
- Ethyl Acrylate and Methyl Methacrylate Copolymer Dispersion
- Ethylcellulose
- Ethylcellulose Aqueous Dispersion
- Ethylcellulose Dispersion Type B
- Pullulan

**Polymers for Ophthalmic Use**

**Dosage Form:** Ophthalmic Preparations
- Carbomer Copolymer
- Carbomer Homopolymer
- Carbomer Interpolymer
- Carmellose
- Guar Gum
- Hydroxyethyl Cellulose
- Hypermellose
- Polyvinyl Alcohol
- Povidone
- Xanthan Gum

**Propellant**

**Dosage Form:** Aerosols
- Butane
- Carbon Dioxide
- Dichlorodifluoromethane
- Dichlorotetrafluoroethane
- Isobutane
- Nitrogen
- Nitrous Oxide
- Propane
- Trichloromonofluoromethane

**Reducing Agent**

**Dosage Form:** Radiopharmaceuticals
- Stannous Chloride
- Stannous Fluoride

**Release-Modifying Agent**

**Dosage Form:** Tablets and Capsules
- Alginic Acid
- Carbomer Copolymer
- Carbomer Homopolymer
- Carbomer Interpolymer
- Carboxymethylcellulose Sodium
- Carrageenan
- Cellaburate
- Ethylcellulose
- Ethylcellulose Aqueous Dispersion
- Ethylcellulose Dispersion Type B
- Glyceryl Monoooleate
- Glyceryl Monostearate
- Guar Gum
- Hydroxypropyl Betadex
- Hydroxypropyl Cellulose
- Hypermellose
- Polyethylene Oxide
- Polyvinyl Acetate Dispersion
- Shellac
<table>
<thead>
<tr>
<th>Sodium Alginate</th>
<th>Polypropylene Glycol 11 Stearyl Ether</th>
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</thead>
<tbody>
<tr>
<td>Starch, Pregelatinized</td>
<td>Propylene Carbonate</td>
</tr>
<tr>
<td>Starch, Pregelatinized Modified</td>
<td>Propylene Glycol</td>
</tr>
<tr>
<td>Xanthan Gum</td>
<td>Propylene Glycol Diacetate</td>
</tr>
<tr>
<td>Sequestering Agent</td>
<td>Safflower Oil</td>
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<tr>
<td><strong>Dosage Form:</strong> Oral Liquids</td>
<td>Sesame Oil</td>
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<tr>
<td>Betadex</td>
<td>Soybean Oil</td>
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<tr>
<td>Betadex Sulfobutyl Ether Sodium</td>
<td>Stearoyl Polyoxyglycerides</td>
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<td>Calcium Acetate</td>
<td>Sunflower Oil</td>
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<tr>
<td>Cyclodextrin, Gamma</td>
<td>Triacetin</td>
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<tr>
<td>Hydroxypropyl Betadex</td>
<td>Triethyl Citrate</td>
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<tr>
<td>Pentetic Acid</td>
<td>Water for Injection</td>
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<tr>
<td>Pullulan</td>
<td>Water for Injection, Sterile</td>
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<tr>
<td>Sodium Citrate</td>
<td>Water for Irrigation, Sterile</td>
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<td>Solvent</td>
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<td>Benzyl Benzoate</td>
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<td>Butyl Alcohol</td>
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<td>Butylene Glycol</td>
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<td>Caprylocaproyl Polyoxyglycerides</td>
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<td>Castor Oil</td>
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<td>Corn Oil</td>
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<tr>
<td>Cottonseed Oil</td>
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<td>Dibutyl Phthalate</td>
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<td>Diethyl Phthalate</td>
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<td>Diethylene Glycol Monoethyl Ether</td>
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<td>Ethyl Oleate</td>
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<td>Glycerin</td>
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<td>Linoleoyl Polyoxyglycerides</td>
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<td>Methylpyrrolidone</td>
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<td>Mineral Oil</td>
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<td>Oleoyl Polyoxyglycerides</td>
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<td>Peanut Oil</td>
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<tr>
<td>Polydecene, Hydrogenated</td>
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<tr>
<td>Polyethylene Glycol</td>
<td></td>
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<tr>
<td>Polyethylene Glycol 3350</td>
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</tr>
<tr>
<td>Polyethylene Glycol Monomethyl Ether</td>
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</tr>
</tbody>
</table>

**Sorbent**

Cellulose, Powdered
Charcoal, Activated
Siliceous Earth, Purified

**Sorbent, Carbon Dioxide**

Barium Hydroxide Lime
Soda Lime

**Stiffening Agent**

**Dosage Form:** Semisolids, Topicals, and Suppositories

Castor Oil, Hydrogenated
Cetostearyl Alcohol
Cetyl Alcohol
Cetyl Palmitate
Dextrin
Hard Fat
Alpha-Lactalbumin
Paraffin
Paraffin, Synthetic
Rapeseed Oil, Fully Hydrogenated
Rapeseed Oil, Superglycerinated Fully Hydrogenated
Sodium Stearate
Stearyl Alcohol
Wax, Cetyl Esters
Wax, Emulsifying
Wax, Microcrystalline
Wax, White
Wax, Yellow

**Suppository Base**

**Dosage Form:** Semisolids, Topicals, and Suppositories

Agar
Cocoa Butter
Hard Fat
Palm Kernel Oil
Polyethylene Glycol
Polyethylene Glycol 3350
Suspending and/or Viscosity-Increasing Agent

**Dosage Form:** Semisolids, Topicals, and Suppositories

- Acacia
- Agar
- Alamic Acid
- Alginic Acid
- Aluminum Monostearate
- Attapulgite, Activated
- Attapulgite, Colloidal Activated
- Bentonite
- Bentonite, Purified
- Bentonite Magma
- Carbomer 910
- Carbomer 934
- Carbomer 934P
- Carbomer 940
- Carbomer 941
- Carbomer 1342
- Carbomer Copolymer
- Carbomer Homopolymer
- Carbomer Interpolymer
- Carboxymethylcellulose Calcium
- Carboxymethylcellulose Sodium
- Carboxymethylcellulose Sodium 12
- Carboxymethylcellulose Sodium, Enzymatically-Hydrolyzed
- Carmellose
- Carrageenan
- Cellulose, Microcrystalline
- Cellulose, Microcrystalline, and Carboxymethylcellulose Sodium
- Cellulose, Powdered
- Cetostearyl Alcohol
- Chitosan
- Corn Syrup
- Corn Syrup Solids
- Cyclomethicone
- Dextrin
- Egg Phospholipids
- Ethylcellulose
- Gelatin
- Gellan Gum
- Glyceryl Behenate
- Methylcellulose
- Pectin
- Polycarbophil
- Polydextrose
- Polydextrose, Hydrogenated
- Polyethylene Oxide
- Polysorbate 20
- Polysorbate 40
- Polysorbate 60
- Polysorbate 80
- Polyvinyl Alcohol
- Potassium Alginate
- Povidone
- Propylene Glycol Alginate
- Pullulan
- Silica, Dental-Type
- Silica, Hydrophobic Colloidal
- Silicon Dioxide
- Silicon Dioxide, Colloidal
- Sodium Alginate
- Sorbitan Monolaurate
- Sorbitan Monoooleate
- Sorbitan Monopalmitate
- Sorbitan Monostearate
- Sorbitan Sesquioleate
- Sorbitan Trioleate
- Starch, Corn
- Starch, Hydroxypropyl Corn
- Starch, Pregelatinized Hydroxypropyl Corn
- Starch, Pea
- Starch, Hydroxypropyl Pea
- Starch, Pregelatinized Hydroxypropyl Pea
- Starch, Potato
- Starch, Hydroxypropyl Potato
- Starch, Pregelatinized Hydroxypropyl Potato
- Starch, Tapioca
- Starch, Wheat
- Sucrose
- Sucrose Palmitate
- Tragacanth
- Vitamin E Polyethylene Glycol Succinate
- Xanthan Gum

Sweetening Agent

**Dosage Form:** Oral Liquids

- Acesulfame Potassium
- Aspartame
- Aspartame Acesulfame
- Corn Syrup
- Corn Syrup, High Fructose
- Corn Syrup Solids
- Dextrates
- Dextrose
- Dextrose Excipient
- Erythritol
- Fructose
- Galactose
- Glucose, Liquid
- Glycerin
- Inulin
Invert Sugar
Isomalt
Lactitol
Maltitol
Maltitol Solution
Maltose
Mannitol
Neotame
Neohesperidin Dihydrochalcone
Saccharin
Saccharin Calcium
Saccharin Sodium
Sorbitol
Sorbitol Solution
Starch Hydrolysate, Hydrogenated
Sucralose
Sucrose
Sugar, Compressible
Sugar, Confectioner’s Syrup
Tagatose
Trehalose
Xylitol

**Tonicity Agent**

**Dosage Form:** Parenterals
- Corn Syrup
- Corn Syrup Solids
- Dextrose
- Glycerin
- Mannitol
- Potassium Chloride
- Sodium Chloride

**Transfer Ligand**

**Dosage Form:** Radiopharmaceuticals
- Edetate Disodium
- Sodium Acetate
- Sodium Citrate
- Sodium Gluconate
- Sodium Tartrate

**Vehicle**

Polypropylene Glycol 11 Stearyl Ether

**Dosage Form:** Oral Liquids

**FLAVORED AND/OR SWEETENED**
- Aromatic Elixir
- Benzaldehyde Elixir, Compound
- Corn Syrup Solids
- Dextrose
- Ethyl Maltol
- Peppermint Water
- Sorbitol Solution
- Syrup
- Trehalose

**OLEAGINOUS**
- Alkyl (C12–15) Benzoate
- Almond Oil
- Canola Oil

- Castor Oil
- Corn Oil
- Cottonseed Oil
- Ethyl Oleate
- Isopropyl Myristate
- Isopropyl Palmitate
- Mineral Oil
- Mineral Oil, Light
- Myristyl Alcohol
- Octyldodecanol
- Olive Oil
- Peanut Oil
- Polydecene, Hydrogenated
- Polyoxy 15 Hydroxystearate
- Safflower Oil
- Sesame Oil
- Soybean Oil
- Squalane
- Sunflower Oil

**SOLID CARRIER**
- Chitosan
- Corn Syrup Solids
- Alpha-Lactalbumin
- Glyceryl Tricaprylate
- Propylene Glycol Dicaprylate/Dicaprate
- Propylene Glycol Monocaprylate
- Sugar Spheres

**STERILE**
- Albumin Human
- Sodium Chloride Injection, Bacteriostatic
- Water for Injection, Bacteriostatic

**Water-Repelling Agent**
- Cyclomethicone
- Dimethicone
- Simethicone

**Change to read:**

**Wet Binder**

**Dosage Form:** Tablets and Capsules
- Acacia
- Agar
- Alginic Acid
- Amino Methacrylate Copolymer
- Ammonio Methacrylate Copolymer
- Ammonio Methacrylate Copolymer Dispersion
- Calcium Carbonate
- Calcium Lactate
- Carbomer Copolymer
- Carbomer Homopolymer
- Carbomer Interpolymer
- Carboxymethylcellulose Sodium
- Cellulose, Microcrystalline
- Cellulose, Silicified Microcrystalline
- Coconut Oil, Hydrogenated
- Copovidone
- Corn Syrup
- Corn Syrup Solids

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Wetting and/or Solubilizing Agent

Betadex Sulfobutyl Ether Sodium

**Dosage Form:** Oral Liquids

Behenyl Polyoxyglycerides
Benzalkonium Chloride
Benzethonium Chloride
Butylene Glycol
Caprylocaproyl Polyoxyglycerides
Cetylpyridinium Chloride
Docucate Sodium
Egg Phospholipids
Glycine
Lauroyl Polyoxyglycerides
Linoleoyl Polyoxyglycerides
Nonoxynol 9
Octoxynol 9
Oleoyl Polyoxyglycerides
Poloxamer
Polyoxyl 10 Oleyl Ether
Polyoxy 15 Hydroxystearate
Polyoxy 20 Cetostearyl Ether
Polyoxy 35 Castor Oil
Polyoxy 40 Castor Oil, Hydrogenated
Polyoxy 40 Stearate
Polyoxy Lauryl Ether
Polyoxy Stearate
Polyoxy Stearyl Ether
Polysorbate 20
Polysorbate 40
Polysorbate 60
Polysorbate 80
Pullulan
Sodium Lauryl Sulfate
Sorbitan Monolaurate
Sorbitan Monoooleate
Sorbitan Monopalmitate
Sorbitan Monostearate
Sorbitan Sesquioleate
Sorbitan Trioleate
Stearoyl Polyoxyglycerides
Tyloxapol
Wax, Emulsifying
NF Monographs

Ammonium Glycyrrhizate

C₂₅H₂₁O₁₈·NH₃  840.08
Monoammonium glycyrrhizinate;
Glycyrrhizic acid ammonium salt;
α-D-Glucopyranosiduronic acid, (3β,20β)-20-carboxy-11-oxo-30-norolean-12-en-3-yl 2-O-β-D-glucopyranuronosyl-,
ammonium salt (1:1);
α-D-Glucopyranosiduronic acid, (3β,20β)-20-carboxy-11-oxo-30-norolean-12-en-3-yl 2-O-β-D-glucopyranuronosyl-,
monoammonium salt  [53956-04-0].

DEFINITION
Ammonium Glycyrrhizate is a mixture of ammonium 18α- and
18β-glycyrrhizate (ammonium salt of (20β)-
3β-[2-O-(β-D-glucopyranosyluronic
acid)-α-D-glucopyranosyluronic acid][oxy]-11-oxoolean-12-en-29-oic acid), and the 18β-isomer is the main component. It contains NLT 78.0% and NMT 102.0% of ammonium 18α- and 18β-glycyrrhizate, on the anhydrous basis.

IDENTIFICATION
• A. The retention times of the peaks of 18α- and
18β-glycyrrhizic acid from the Sample solution correspond to those from the System suitability solution, as obtained in the Content of Ammonium 18α- and 18β-Glycyrrhizate.
[NOTE—The peak of 18α-glycyrrhizic acid could be absent in the Sample solution.]
• B. IDENTIFICATION TESTS—GENERAL, Ammonium (191)
Acceptance criteria: Meets the requirements

ASSAY
• CONTENT OF AMMONIUM 18α- AND 18β-GLYCYRRHIZATE
Mobile phase: Acetonitrile, glacial acetic acid, and water
(38:1:61)
Standard solution: 0.5 mg/mL of USP Glycyrrhizic Acid RS
in Mobile phase
System suitability solution: 0.5 mg/mL of USP Ammonium
Glycyrrhizate RS in Mobile phase
Sample solution: 0.5 mg/mL of Ammonium Glycyrrhizate
in Mobile phase
Chromatographic system
(See Chromatography (621), System Suitability.)
Mode: LC
Detector: UV 254 nm
Column: 3.9-mm x 30-cm analytical column; 5–10 μm
packing L1
Flow rate: 2.0 mL/min
Injection volume: 10 μL
System suitability
Samples: System suitability solution and Standard solution
[NOTE—The relative retention times of 18β-glycyrrhizic
acid and 18α-glycyrrhizic acid are about 1.0 and 1.2,
respectively, System suitability solution.]
Suitability requirements
Resolution: NLT 2.0 between the peaks due to
18β-glycyrrhizic acid and 18α-glycyrrhizic acid, System
suitability solution
Relative standard deviation: NMT 2.0%, Standard
solution
Analysis
Samples: Standard solution, System suitability solution, and
Sample solution
Determine the peak areas for each isomer
(18α-glycyrrhizic acid or 18β-glycyrrhizic acid).
Calculate the percentage of ammonium 18α-glycyrrhizate
(or ammonium 18β-glycyrrhizate) in the portion of Ammonium Glycyrrhizate taken:

Result = \( \frac{r_u}{r_s} \times \frac{C_s}{C_u} \times \left( \frac{M_{\text{WSalt}}}{M_{\text{WAcid}}} \right) \times 100 \)

\( r_u \) = peak area of the 18α-glycyrrhizic acid (or
18β-glycyrrhizic acid) in the Sample solution
\( r_s \) = peak area of the 18β-glycyrrhizic acid in the
Standard solution
\( C_u \) = concentration of the Standard solution
Glycyrrhizic Acid RS in the Standard solution (mg/mL)
\( C_s \) = concentration of the Sample solution (mg/mL)
\( M_{\text{WSalt}} \) = molecular weight of ammonium glycyrrhizate,
840.08 g/mol
\( M_{\text{WAcid}} \) = molecular weight of glycyrrhizic acid,
821.59 g/mol

Acceptance criteria: The total percentage of ammonium
18α-glycyrrhizate and ammonium 18β-glycyrrhizate is
78.0%–102.0%, and the percentage of ammonium
18α-glycyrrhizate is NMT 13.0%, on the anhydrous basis.

IMPURITIES
• RESIDUE ON IGNITION (281)
Sample: 1.0 g
Acceptance criteria: NMT 0.5%
• LIMIT OF ORGANIC IMPURITIES
Mobile phase, System suitability solution, and
Chromatographic system: Proceed as directed in Content
of Ammonium 18α- and 18β-Glycyrrhizate.
Sample solution: 1.0 mg/mL of Ammonium Glycyrrhizate
in the Mobile phase
Reference solution A: 0.05 mg/mL of Ammonium Glycyrrhizate
in the Mobile phase, prepared from the
Sample solution
Reference solution B: 0.057 mg/mL of Ammonium Glycyrrhizate
in the Mobile phase, prepared from the
Sample solution
System suitability
Sample: System suitability solution
[NOTE—The relative retention times for
24-hydroxyglycyrrhizic acid, 18β-glycyrrhizic
acid, and 18α-glycyrrhetic acid are about 0.7, 1.0 and 1.2, respectively.

Suitability requirements
Resolution: NLT 2.0 between the peaks due to 18β-glycyrrhetic acid and 18α-glycyrrhetic acid

Analysis
Samples: System suitability solution, Reference solution A, Reference solution B, and Sample solution

Acceptance criteria: See Table 1.

<table>
<thead>
<tr>
<th>Name</th>
<th>Relative Retention Time</th>
<th>Acceptance Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>24-Hydroxy-glycyrrhetic acidα</td>
<td>0.7</td>
<td>NMT the sum of the areas of the peaks in the chromatogram from Reference solution B, corresponding to NMT 5.7%</td>
</tr>
<tr>
<td>Any other impurity</td>
<td></td>
<td>For each impurity, NMT 0.4 times the sum of the areas of the peaks in the chromatogram from Reference solution A, corresponding to NMT 2.0%</td>
</tr>
<tr>
<td>Sum of other impurities</td>
<td></td>
<td>NMT 1.6 times the sum of the areas of the peaks in the chromatogram from Reference solution A, corresponding to NMT 8.0%</td>
</tr>
</tbody>
</table>

SPECIFIC TESTS
• OPTICAL ROTATION, Specific Rotation (781)
  Sample solution: 10.0 mg/mL of Ammonium Glycyrrhizate in 50% ethanol
  Acceptance criteria: +49.0 to +55.0 on the anhydrous basis

• WATER DETERMINATION, Method Ia (921)
  Sample: 0.25 g
  Acceptance criteria: NMT 6.0%

ADDITIONAL REQUIREMENTS
• PACKAGING AND STORAGE: Preserve in tight containers, and store in a cool, dry place.
• USP REFERENCE STANDARDS (11)
  USP Ammonium Glycyrrhizate RS
  USP Glycyrrhizic Acid RS

Table 1 (continued)

<table>
<thead>
<tr>
<th>Name</th>
<th>Relative Retention Time</th>
<th>Acceptance Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disregard limit</td>
<td></td>
<td>0.04 times the sum of the areas of the peaks in the chromatogram from Reference solution A, corresponding to 0.2%</td>
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</table>

a (4β,20β)-3β-(2-O-(β-D-Glucopyranosyluronic acid)-β-D-glucopyranosyluronic acid)oxy)-23-hydroxy-11-oxoolean-12-en-29-oic acid.
CONTAINERS FOR DISPENSING CAPSULES AND TABLETS

The following table is provided as a reminder for the pharmacist engaged in the typical dispensing situation who already is acquainted with the Packaging and Storage requirements set forth in the individual monographs. It lists the capsules and tablets that are official in the United States Pharmacopeia and indicates the relevant tight (T), well-closed (W), and light-resistant (LR) specifications applicable to containers in which the drug that is repackaged should be dispensed.

This table is not intended to replace, nor should it be interpreted as replacing, the definitive requirements stated in the individual monographs.

<table>
<thead>
<tr>
<th>Container Specifications for Capsules and Tablets</th>
<th>Container Specifications for Capsules and Tablets (continued)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Monograph Title</strong></td>
<td><strong>Container Specification</strong></td>
</tr>
<tr>
<td>Abacavir Tablets</td>
<td>W</td>
</tr>
<tr>
<td>Abacavir and Lamivudine Tablets</td>
<td>T, LR</td>
</tr>
<tr>
<td>Abiraterone Acetate Tablets</td>
<td>T</td>
</tr>
<tr>
<td>Acarbose Tablets</td>
<td>T, LR</td>
</tr>
<tr>
<td>Acetaminophen Capsules</td>
<td>T</td>
</tr>
<tr>
<td>Acetaminophen Tablets, Extended-Release</td>
<td>T</td>
</tr>
<tr>
<td>Acetaminophen Tablets</td>
<td>T</td>
</tr>
<tr>
<td>Acetaminophen and Aspirin Tablets</td>
<td>T</td>
</tr>
<tr>
<td>Acetaminophen, Aspirin, and Caffeine Tablets</td>
<td>T</td>
</tr>
<tr>
<td>Acetaminophen and Caffeine Tablets</td>
<td>T</td>
</tr>
<tr>
<td>Acetaminophen and Salts of Chlorpheniramine, Dextromethorphan, and Pseudoephedrine, Capsules Containing at Least Three of the Following—</td>
<td>T</td>
</tr>
<tr>
<td>Acetaminophen, Chlorpheniramine Maleate, and Dextromethorphan Hydrobromide Tablets</td>
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</tr>
<tr>
<td>Acetaminophen and Codeine Phosphate Capsules</td>
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<tr>
<td>Acetaminophen and Codeine Phosphate Tablets</td>
<td>T, LR</td>
</tr>
<tr>
<td>Acetaminophen and Diphenhydramine Citrate Tablets</td>
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<tr>
<td>Acetaminophen, Diphenhydramine Hydrochloride, and Pseudoephedrine Hydrochloride Tablets</td>
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</tr>
<tr>
<td>Acetaminophen and Pseudoephedrine Hydrochloride Tablets</td>
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<tr>
<td>Acetaminophen and Tramadol Hydrochloride Tablets</td>
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</tr>
<tr>
<td>Acetazolamide Tablets</td>
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<td>Container Specification</td>
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<tr>
<td>-------------------------------------------------------------------------------</td>
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<td>Aminophylline Tablets, Delayed-Release</td>
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<td>Amiodarone Hydrochloride Tablets</td>
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<td>Amitriptyline Hydrochloride Tablets</td>
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<tr>
<td>Amlodipine and Atorvastatin Tablets</td>
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<td>Amlodipine and Benazepril Hydrochloride Capsules</td>
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<td>Amlodipine Besylate Tablets</td>
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<td>Amlodipine and Olmesartan Medoxomil Tablets</td>
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<tr>
<td>Amlodipine and Valsartan Tablets</td>
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<td>Amlodipine, Valsartan, and Hydrochlorothiazide Tablets</td>
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<td>Amodiaquine Hydrochloride Tablets</td>
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<td>Amoxapine Tablets</td>
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<td>Amoxicillin and Clavulanic Acid Tablets, Extended-Release</td>
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<td>Aspirin Tablets, Effervescent for Oral Solution</td>
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<td>Aspirin, Alumina, and Magnesium Tablets</td>
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<td>Aspirin, Alumina, and Magnesium Oxide Tablets</td>
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<td>Atenolol Tablets</td>
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<tr>
<td>Atenolol and Chlorthalidone Tablets</td>
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</tbody>
</table>

**Delete the following:**

* Aspirin, Caffeine, and Dihydrocodeine Bitartrate Capsules [T (USP 1-Dec-2020)]
* Aspirin and Codeine Phosphate Tablets                                      | W, LR                   |
* Aspirin, Codeine Phosphate, Alumina, and Magnesium Tablets                | W, LR                   |
* Atenolol Tablets                                                           | W                       |
* Atenolol and Chlorthalidone Tablets                                         | W                       |

<table>
<thead>
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<th>Monograph Title</th>
<th>Container Specification</th>
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<td>Atomoxetine Capsules</td>
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<td>Bacillus coagulans Capsules</td>
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<td>Baclofen Tablets</td>
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<td>Bismuth Subsalicylate Tablets</td>
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<td>Bisoprolol Fumarate Tablets</td>
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<td>Borage Seed Oil Capsules</td>
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<tr>
<td>Buprenorphine and Naloxone Sublingual Tablets</td>
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<td>Bupropion Hydrochloride Tablets, Extended-Release</td>
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<tr>
<td>Buspirone Hydrochloride Tablets</td>
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<tr>
<td>Busulfan Tablets</td>
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<td>Butabarbital Sodium Tablets</td>
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<tr>
<td>Butalbital, Acetaminophen, and Caffeine Capsules</td>
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<td>Butalbital, Acetaminophen, and Caffeine Tablets</td>
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<td>Butalbital, Aspirin, and Caffeine Capsules</td>
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<tr>
<td>Butalbital, Aspirin, Caffeine, and Codeine Phosphate Capsules</td>
<td>T, LR</td>
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<td>Cabergoline Tablets</td>
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### Container Specifications for Capsules and Tablets (continued)

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<thead>
<tr>
<th>Monograph Title</th>
<th>Container Specification</th>
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<tbody>
<tr>
<td>Calcifediol Capsules</td>
<td>T, LR</td>
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<tr>
<td>Calcium with Vitamin D Tablets</td>
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<tr>
<td>Calcium Acetate Capsules</td>
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<tr>
<td>Calcium Acetate Tablets</td>
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<tr>
<td>Calcium Carbonate Tablets</td>
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<tr>
<td>Calcium Carbonate and Magnesia Tablets</td>
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<tr>
<td>Calcium Carbonate and Magnesia Chewable Tablets</td>
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<tr>
<td>Calcium Citrate Tablets</td>
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<tr>
<td>Calcium and Magnesium Carbonates</td>
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<td>Calcium Pantothenate Tablets</td>
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<td>Candesartan Cilexetil Tablets</td>
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<td>Captopril Tablets</td>
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<td>Carbamazepine Tablets</td>
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<tr>
<td>Carbamazepine Tablets, Extended-Release</td>
<td>T</td>
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</tbody>
</table>

**Delete the following:**

- *Carbenicillin Indanyl Sodium Tablets* T (USP 1-Dec-2020)

**Carbipoda and Levodopa Tablets**

<table>
<thead>
<tr>
<th>Monograph Title</th>
<th>Container Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbipoda and Levodopa Tablets</td>
<td>T, LR</td>
</tr>
<tr>
<td>Carbipoda and Levodopa Tablets, Extended-Release</td>
<td>W, LR</td>
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<tr>
<td>Carbipoda and Levodopa Tablets, Orally Disintegrating</td>
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<tr>
<td>Carboxymethylcellulose Sodium Tablets</td>
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<tr>
<td>Carisoprodol Tablets</td>
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<td>Carvedilol Tablets</td>
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<td>Cefadroxil Tablets</td>
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</table>

**Chloral Hydrate Capsules**

<table>
<thead>
<tr>
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<th>Container Specification</th>
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<tbody>
<tr>
<td>Chloral Hydrate Capsules</td>
<td>T</td>
</tr>
<tr>
<td>Chlorambucil Tablets</td>
<td>W, LR</td>
</tr>
<tr>
<td>Chloramphenicol Capsules</td>
<td>T</td>
</tr>
<tr>
<td>Chloramphenicol Tablets</td>
<td>T</td>
</tr>
<tr>
<td>Chlordiazepoxide Tablets</td>
<td>T, LR</td>
</tr>
<tr>
<td>Chlordiazepoxide and Amitriptyline Hydrochloride Tablets</td>
<td>T, LR</td>
</tr>
<tr>
<td>Chlordiazepoxide Hydrochloride Capsules</td>
<td>T, LR</td>
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<tr>
<td>Chlordiazepoxide Hydrochloride Capsules</td>
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<tr>
<td>Chloroquine Phosphate Tablets</td>
<td>W</td>
</tr>
<tr>
<td>Chlorothiazide Tablets</td>
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</tr>
<tr>
<td>Chlorpheniramine Maleate Capsules, Extended-Release</td>
<td>T</td>
</tr>
<tr>
<td>Chlorpheniramine Maleate Tablets</td>
<td>T</td>
</tr>
<tr>
<td>Chlorpheniramine Maleate and Pseudoephedrine Hydrochloride Capsules, Extended-Release</td>
<td>T, LR</td>
</tr>
<tr>
<td>Chlorpromazine Hydrochloride Tablets</td>
<td>W, LR</td>
</tr>
<tr>
<td>Chlorpropamide Tablets</td>
<td>W</td>
</tr>
<tr>
<td>Chlorotetracycline Hydrochloride Tablets</td>
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</tr>
<tr>
<td>Chlorothalidone Tablets</td>
<td>W</td>
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<tr>
<td>Chlorzoxazone Tablets</td>
<td>T</td>
</tr>
<tr>
<td>Chlorzoxazone and Acetaminophen Capsules</td>
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<td>Cholecalciferol Capsules</td>
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<td>Cholecalciferol Tablets</td>
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<td>Chondroitin Sulfate Tablets</td>
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<tr>
<td>Chromium Picolinate Tablets</td>
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</tr>
<tr>
<td>Cimetidine Tablets</td>
<td>T, LR</td>
</tr>
<tr>
<td>Cilostazol Tablets</td>
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</tr>
<tr>
<td>Ciprofloxacin Tablets</td>
<td>W</td>
</tr>
<tr>
<td>Ciprofloxacin Tablets, Extended-Release</td>
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</tr>
<tr>
<td>Citalopram Tablets</td>
<td>W</td>
</tr>
<tr>
<td>Clarithromycin Tablets</td>
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**Published on March 26, 2020**
<table>
<thead>
<tr>
<th>Monograph Title</th>
<th>Container Specification</th>
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<tr>
<td>Clarithromycin Tablets, Extended-Release</td>
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</tr>
<tr>
<td>Clemastine Fumarate Tablets</td>
<td>W</td>
</tr>
<tr>
<td>Clindamycin Hydrochloride Capsules</td>
<td>T</td>
</tr>
<tr>
<td>Clofazimine Capsules</td>
<td>W</td>
</tr>
</tbody>
</table>

**Delete the following:**

*Clofibrate Capsules*

- Clomiphene Citrate Tablets                         W, L, R
- Clo mipramine Hydrochloride Capsules               T, LR
- Clonazepam Tablets                                 T, LR
- Clonazepam Tablets, Orally Disintegrating          W, LR
- Clonidine Hydrochloride Tablets                    W
- Clonidine Hydrochloride and Chlorothalidone Tablets W
- Clopidogrel Tablets                                W
- Clorazepate Dipotassium Tablets                    T, LR
- Clotrimazole Tablets, Vaginal                      W
- Red Clover Tablets                                 T, LR
- Cloxacillin Sodium Capsules                        T
- Clozapine Tablets                                  W
- Cyanocobalamin Co 57 Capsules                      W, LR
- Cyanocobalamin Co 58 Capsules                      W, LR
- Cocaine Hydrochloride Tablets for Topical Solution W, LR
- Codeine Phosphate Tablets                          W, LR
- Codeine Sulfate Tablets                            W
- Colestipol Hydrochloride Tablets                   T
- Cortisone Acetate Tablets                          W
- Cromolyn Sodium for Inhalation (in Capsules)       T, LR
- Cryptopcodinium cohni Oil Capsules                 T, LR
- Curcuminoids Capsules                              W, LR
- Curcuminoids Tablets                               W, LR
- Cyanocobalamin Tablets                             T, LR
- Cyclizine Hydrochloride Tablets                    T, LR
- Cyclobenzaprine Hydrochloride Capsules, Extended-Release T, LR
- Cyclobenzaprine Hydrochloride Tablets              W
- Cyclophosphamide Tablets                           T
- Cycloserine Capsules                                T
- Cyclosporine Capsules                              T
- Cyproheptadine Hydrochloride Tablets               W
- Danazol Capsules                                   W
- Dantrolene Sodium Capsules                         W
- Dapsone Tablets                                    W, LR
- Demeclocycline Hydrochloride Tablets               T, LR
- Desipramine Hydrochloride Tablets                  T

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<thead>
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<th>Monograph Title</th>
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<td>T</td>
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<tr>
<td>Desloratadine Tablets, Orally Disintegrating</td>
<td>T</td>
</tr>
<tr>
<td>Desogestrel and Ethinyl Estradiol Tablets</td>
<td>W</td>
</tr>
<tr>
<td>Dexamethasone Tablets</td>
<td>W</td>
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<tr>
<td>Dextroamphetamine Sulfate Tablets</td>
<td>W</td>
</tr>
<tr>
<td>Diazepam Tablets</td>
<td>T, LR</td>
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<tr>
<td>Dichlorphenamide Tablets</td>
<td>W</td>
</tr>
<tr>
<td>Diclofenac Potassium Tablets</td>
<td>T, LR</td>
</tr>
<tr>
<td>Diclofenac Sodium Tablets, Delayed-Release</td>
<td>T, LR</td>
</tr>
<tr>
<td>Diclofenac Sodium Tablets, Extended-Release</td>
<td>W</td>
</tr>
<tr>
<td>Diclofenac Sodium and Misoprostol Delayed-Release Tablets</td>
<td>T</td>
</tr>
<tr>
<td>Dicloxacillin Sodium Capsules</td>
<td>T</td>
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<tr>
<td>Dicyclomine Hydrochloride Capsules</td>
<td>W</td>
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<tr>
<td>Didanosine Capsules, Delayed-Release</td>
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<tr>
<td>Didanosine Tablets for Oral Suspension</td>
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<td>Diethylcarbamazine Citrate Tablets</td>
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<td>Diflunisal Tablets</td>
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<td>Digitalis Capsules</td>
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**Delete the following:**

*Dihydrotachysterol Capsules*  

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<th>Monograph Title</th>
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<td>Dihydrotachysterol Tablets</td>
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**Delete the following:**

*Dihydrotachysterol Tablets*  

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<tr>
<td>Diltiazem Hydrochloride Tablets</td>
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<tr>
<td>Diltiazem Hydrochloride Capsules, Extended-Release</td>
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<tr>
<td>Dimenhydrinate Tablets</td>
<td>W</td>
</tr>
<tr>
<td>Diphenhydramine Citrate and Ibuprofen Tablets</td>
<td>T</td>
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<tr>
<td>Diphenhydramine Hydrochloride Capsules</td>
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<tr>
<td>Diphenhydramine Hydrochloride and Ibuprofen Capsules</td>
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<tr>
<td>Diphenhydramine and Phenylephrine Hydrochlorides Tablets</td>
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<tr>
<td>Diphenhydramine and Pseudoephedrine Capsules</td>
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</tr>
<tr>
<td>Diphenoxylate Hydrochloride and Atropine Sulfate Tablets</td>
<td>W, LR</td>
</tr>
<tr>
<td>Dipyridamole Tablets</td>
<td>T, LR</td>
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<thead>
<tr>
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<th>Container Specification</th>
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<td>Delete the following:</td>
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<td>Disopyramide Phosphate Capsules</td>
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<td>Disopyramide Phosphate Capsules, Extended-Release</td>
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<tr>
<td>Disulfiram Tablets</td>
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<td>Divalproex Sodium Capsules, Delayed-Release</td>
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<td>W</td>
</tr>
<tr>
<td>Docusate Calcium Capsules</td>
<td>T</td>
</tr>
<tr>
<td>Docusate Potassium Capsules</td>
<td>T</td>
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<tr>
<td>Docusate Sodium Tablets</td>
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<td>Donepezil Hydrochloride Tablets</td>
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<tr>
<td>Donepezil Hydrochloride Tablets, Orally Disintegrating</td>
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<td>Doxazosin Tablets</td>
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<td>Doxepin Hydrochloride Capsules</td>
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<td>Doxylamine Succinate Tablets</td>
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<td>Dronabinol Capsules</td>
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<td>Dyphylline and Guaifenesin Tablets</td>
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<tr>
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<td>Echinacea Species Powder Capsules</td>
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<td>Efavirenz Capsules</td>
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<td>Efaviren Tablets</td>
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<td>Eleuthero Root and Rhizome Dry Extract Capsules</td>
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<td>Eleuthero Root and Rhizome Dry Extract Tablets</td>
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<tr>
<td>Eleuthero Root and Rhizome Powder Capsules</td>
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</tr>
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<td>Enalapril Maleate Tablets</td>
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<tr>
<td>Enalapril Maleate and Hydrochlorothiazide Tablets</td>
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<table>
<thead>
<tr>
<th>Monograph Title</th>
<th>Container Specification</th>
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<tr>
<td>▲ Ergoloid Mesylates Capsules</td>
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<td>Ergonovine Maleate Tablets</td>
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<tr>
<td>Ergotamine Tartrate Tablets</td>
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<tr>
<td>Ergotamine Tartrate Tablets, Sublingual</td>
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<td>Ergotamine Tartrate and Caffeine Tablets</td>
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<tr>
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<td>Erythromycin Ethylsuccinate Tablets</td>
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<td>Erythromycin Stearate Tablets</td>
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<td>Escalopram Tablets</td>
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<td>Esomeprazole Magnesium Capsules, Delayed-Release</td>
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<td>Estazolam Tablets</td>
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<tr>
<td>Estradiol Tablets</td>
<td>T, LR</td>
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<tr>
<td>Estradiol and Norethindrone Acetate Tablets</td>
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<td>Estrogens Tablets, Conjugated</td>
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<td>Estrogens Tablets, Esterified</td>
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<td>Eszopiclone Tablets</td>
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<td>Ethacrynic Acid Tablets</td>
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<td>Delete the following:</td>
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<td>Etothoin Tablets</td>
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<td>Ethynodiol Diacetate and Ethinyl Estradiol Tablets</td>
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<td>Ethynodiol Diacetate and Mestranol Tablets</td>
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<td>Etodolac Tablets, Extended-Release</td>
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</tr>
<tr>
<td>Evening Primrose Oil Capsules</td>
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</table>
### Container Specifications for Capsules and Tablets (continued)

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<thead>
<tr>
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<tbody>
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<td>Famciclovir Tablets</td>
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<td>Famotidine Tablets</td>
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<td>Felbamate Tablets</td>
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<td>Felodipine Tablets, Extended-Release</td>
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<td>Fenofibrate Tablets</td>
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<td>Fenoprofen Calcium Capsules</td>
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<td>Ferrous Fumarate and Docusate Sodium Tablets, Extended-Release</td>
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<td>Fluconazole Tablets</td>
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<td>Flucytosine Capsules</td>
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<td>Fluoxetine Capsules, Delayed-Release</td>
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<td>Fosinopril Sodium and Hydrochlorothiazide Tablets</td>
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</tbody>
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**Delete the following:**

- Furazolidone Tablets
- Furosemide Tablets W, LR

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### Container Specifications for Capsules and Tablets (continued)

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<th>Container Specification</th>
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Published on March 26, 2020
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DELETE THE FOLLOWING:

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Sulfamethoxazole and Trimethoprim Tablets  
Sulfapyridine Tablets  

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## Container Specifications for Capsules and Tablets (continued)

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**Not official text. Please refer to the currently official version of the applicable USP–NF or FCC standard for compliance purposes.**

Published on March 26, 2020
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Published on March 26, 2020.
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Not official text. Please refer to the currently official version of the applicable USP–NF or FCC standard for compliance purposes.

Published on March 26, 2020
General Tests and Assays

(1) INJECTIONS AND IMPLANTED DRUG PRODUCTS (PARENTERALS)—PRODUCT QUALITY TESTS

INTRODUCTION

PRODUCT QUALITY TESTS COMMON TO PARENTERAL DOSAGE FORMS
- Universal Tests
- Specific Tests

PRODUCT QUALITY TESTS FOR SPECIFIC PARENTERAL DOSAGE FORMS
- Solutions
- Sterile Powders for Solutions
- Suspensions
- Liposomes
- Sterile Powders for Suspensions
- Emulsions
- Implants
- Drug-Eluting Stents

INTRODUCTION

Parenteral drug products include both injections and implanted drug products that are injected through the skin or other external boundary tissue, or implanted within the body to allow the direct administration of the active drug substance(s) into blood vessels, organs, tissues, or lesions. Injections may exist as either immediate- or extended-release dosage forms. Implanted parenteral drug products are long-acting dosage forms that provide continuous release of the active drug substance(s), often for periods of months to years. For systemic delivery, they may be placed subcutaneously; for local delivery, they may be placed in a specific region of the body. Routes of administration for parenteral drug products include intravenous, intraventricular, intra-arterial, intra-articular, subcutaneous, intramuscular, intrathecal, intracisternal, and intraocular.

Parenteral dosage forms include solutions, suspensions, emulsions, sterile powders for solutions and suspensions (including liposomes), implants (including microparticles), and products that consist of both a drug and a device such as drug-eluting stents. The definitions and descriptions of these dosage forms, and brief information about their composition and manufacturing processes, are found in *Pharmaceutical Dosage Forms* (1151). [NOTE—All references to chapters above 1000 are for informational purposes only, for use as a helpful resource. These chapters are not mandatory unless explicitly called out for application.]

This chapter is divided into three main sections: (1) universal product quality tests that are applicable to parenteral dosage forms; (2) specific product quality tests, which are tests that should be considered in addition to Universal Tests; and (3) product quality tests for specific dosage forms, which list applicable tests (universal and specific) for the specific dosage form.

This chapter applies, in whole or in part, when referenced in a drug product monograph (see General Notices, 3.10 Applicability of Standards).

The pharmacopeial definitions for sterile preparations for parenteral use may not apply to some biologics because of their special nature and licensing requirements (see Biologics (1041)). However, some biological finished drug products containing “Injection” in the monograph title must meet the requirements of (1) or indicated chapter subparts, where it is specified in the monograph.

Change to read:

PRODUCT QUALITY TESTS COMMON TO PARENTERAL DOSAGE FORMS

Universal Tests

Universal tests are listed below and are applicable to parenteral dosage forms.
IDENTIFICATION

Identification tests are discussed in General Notices, 5.40 Identification should establish the identity of the drug or drugs present in the article and should discriminate between compounds of closely related structure that are likely to be present.

ASSAY

A specific and stability-indicating test should be used to determine the strength (content) of the drug product. In cases where the use of a nonspecific assay is justified, other supporting analytical procedures should be used to achieve overall specificity. A specific procedure should be used when there is evidence of excipient interference with the nonspecific assay.

IMPURITIES

Tests for Impurities are discussed in General Notices, 5.60 Impurities and Foreign Substances. All articles should be tested to ensure that they meet the requirements.

FOREIGN AND PARTICULATE MATTER

Articles intended for parenteral administration should be prepared in a manner designed to exclude particulate matter as defined in Subvisible Particulate Matter in Therapeutic Protein Injections (787), Particulate Matter in Injections (788), or Particulate Matter in Ophthalmic Solutions (789), as well as excluding other foreign matter as appropriate for the dosage form. Each final container of all parenteral preparations should be inspected to the extent possible for the presence of observable foreign and particulate matter (hereafter termed visible particulates) in its contents. The inspection process should be designed and qualified to ensure that every lot of all parenteral preparations is essentially free from visible particulates, as defined in Visible Particulates in Injections (790). Qualification of the inspection process should be performed with reference to particulates in the visible range and those particulates that might emanate from the manufacturing or filling process. Every container in which the contents show evidence of visible particulates must be rejected. The inspection for visible particulates may take place during examination for other defects such as cracked or defective containers or seals, or when characterizing the appearance of a lyophilized product. When the nature of the contents or the container–closure system permits only limited inspection of the total contents, the 100% inspection of a lot should be supplemented with the inspection of constituted (e.g., dried) or withdrawn (e.g., from a dark amber container) contents of a sample of containers from the lot.

Large-volume injections for single-dose infusion, small-volume injections, and pharmacy bulk packages (PBPs) are subject to the light obscuration or microscopic procedures and limits for subvisible particulate matter set forth in (788), unless otherwise specified in the chapter or in the individual monograph. An article packaged as both a large-volume and a small-volume injection meets the requirements set forth for small-volume injections where the container is labeled as containing 100 mL or less. It meets the requirements set forth for large-volume injections for single-dose infusion where the container is labeled as containing more than 100 mL.

STERILITY

The sterility of all drug products intended for parenteral administration should be confirmed by the use of methods described in Sterility Tests (71) or by an approved alternative method.

BACTERIAL ENDOTOXINS

All articles intended for parenteral administration should be prepared in a manner designed to limit bacterial endotoxins as defined in Bacterial Endotoxins Test (85) or Pyrogen Test (151).

CONTAINER CONTENT

Container contents should be determined when appropriate (see Container Content for Injections (697)).

PACKAGING SYSTEMS

The packaging system should not interact physically or chemically with the preparation to alter its strength, quality, or purity beyond the official or established requirements. The packaging system should meet the requirements in Plastomeric Components Used in Injectable Pharmaceutical Packaging/Delivery Systems (381). Packaging and Storage Requirements (659), Containers—Glass (660), Plastic Packaging Systems and their Materials of Construction (661), Plastic Materials of Construction (661.1), and Plastic Packaging Systems for Pharmaceutical Use (661.2). Further information regarding packaging systems testing may be found in Assessment of Extractables Associated with Pharmaceutical Packaging/Delivery Systems (1663) and Assessment of Drug Product Leachables Associated with Pharmaceutical Packaging/Delivery Systems (1664).

CONTAINER–CLOSURE INTEGRITY

The packaging system should be closed or sealed in such a manner as to prevent contamination or loss of contents. Validation of container integrity must demonstrate no penetration of microbial contamination or gain or loss of any chemical or physical parameter deemed necessary to protect the product (see Package Integrity Evaluation—Sterile Products (1207), Package Integrity Testing in the Product Life Cycle—Test Method Selection and Validation (1207.1), Package Integrity Leak Test Technologies (1207.2), and Package Seal Quality Test Technologies (1207.3)).
LABELING

All articles intended for parenteral administration should meet the labeling requirements defined in Labeling (7).

Specific Tests

In addition to the Universal Tests listed above, the following specific tests may be necessary depending on the dosage form.

UNIFORMITY OF DOSAGE UNITS

This test is applicable for parenteral drug products and dosage forms packaged in single-unit containers. It includes both the mass of the dosage form and the content of the active substance in the dosage form (see Uniformity of Dosage Units (905)).

VEHICLES AND ADDED SUBSTANCES

There are other vehicles, both aqueous and nonaqueous, beyond those that are discussed below. All vehicles should be suitable for their intended use and not impact drug product quality.

Aqueous vehicles: Aqueous vehicles must meet the requirements of (151) or (85), whichever is specified in the monograph. Water for injection is generally used as the vehicle. Sodium chloride or dextrose may be added to render the resulting solution isotonic, and sodium chloride injection or Ringer’s injection may be used in whole or in part instead of water for injection.

Nonaqueous vehicles: Fixed oils are classified under Nonaqueous vehicles. Fixed oils used as vehicles are of vegetable origin and are odorless. They meet the requirements in the test for Solid Paraffin in the Mineral Oil monograph with the cooling bath maintained at 10°.

Nonaqueous vehicles should also meet the requirements of the following tests:

- Fats and Fixed Oils (401), Saponification Value: Between 185 and 200
- Fats and Fixed Oils (401), Iodine Value: Between 79 and 141
- Fats and Fixed Oils (401), Unsaponifiable Matter: NMT 1.5%  
- Fats and Fixed Oils (401), Acid Value: NMT 0.2  
- Fats and Fixed Oils (401), Peroxide Value: NMT 5.0  
- Water Determination (921), Method Ic: NMT 0.1%  
- Limit of Copper, Iron, Lead, and Nickel: [NOTE—The test for nickel is not required if the oil has not been subjected to hydrogenation, or a nickel catalyst has not been used in processing.] Proceed as directed in Fats and Fixed Oils (401), Trace Metals or Elemental Impurities—Procedures (233). Meet the requirements in Elemental Impurities—Limits (232).

Synthetic mono- or diglycerides of fatty acids may be used provided they are liquid and remain clear when cooled to 10° and have a Iodine Value of NMT 140.

Added substances: Suitable substances may be added to preparations in order to increase stability or usefulness unless they are proscribed in the monograph. No coloring agent may be added to a preparation solely for the purpose of coloring the added substances.

Antimicrobial agents must be added to preparations intended for injection that are packaged in multiple-dose containers unless one of the following conditions prevails: (1) there are different directions in the individual monograph; (2) the substance contains a radionuclide with a physical half-life of less than 24 h; or (3) the active ingredients are themselves antimicrobial. Substances must meet the requirements of (51) and Antimicrobial Agents—Content (341).

ANTIMICROBIAL PRESERVATIVES

Antimicrobial agents must be added to preparations intended for injection that are packaged in multiple-dose containers unless one of the following conditions prevails: (1) there are different directions in the individual monograph; (2) the substance contains a radionuclide with a physical half-life of less than 24 h; or (3) the active ingredients are themselves antimicrobial. Substances must meet the requirements of (51) and Antimicrobial Agents—Content (341).

WATER CONTENT

The water content of freeze-dried (lyophilized) products should be determined when appropriate (see (921)).

ALUMINUM CONTENT

See Labeling (7), Aluminum in Large-Volume Injections (LVIs), Small-Volume Injections (SVIs), and Pharmacy Bulk Packages (PBPs) Used in Parenteral Nutrition (PN) Therapy for information related to specific labeling requirements associated with aluminum content.

COMPLETENESS AND CLARITY OF SOLUTIONS

The following tests are performed to demonstrate suitability of constituted solutions prepared before administration. Constitute the solution as directed in the labeling supplied by the manufacturer:

- The solid dissolves completely, leaving no undissolved matter.
The constituted solution is not significantly less clear than an equal volume of the diluent or of purified water contained in a similar vessel and examined similarly. Protein solutions may exhibit an inherent opalescence. The constituted solution is free from particulate matter that can be observed on visual inspection (see \( \text{aq} \)).

**PRODUCT QUALITY TESTS FOR SPECIFIC PARENTERAL DOSAGE FORMS**

Product quality tests for the specific dosage forms are listed below. Specific chapter(s) referenced for the test can be found in the *Universal Tests* and *Specific Tests* sections.

**Solutions**

A solution is a clear, homogeneous liquid dosage form that contains one or more chemical substances (e.g., drug substances or excipients) dissolved in a solvent (aqueous or nonaqueous) or a mixture of mutually miscible solvents. Solutions intended for parenteral administration (e.g., by injection or for irrigation) must be sterile and biocompatible with the intended administration site. This includes consideration of factors such as tonicity, pH, pyrogenicity, extraneous particulate matter, and physicochemical compatibility, among others.

Unless otherwise justified, the following tests are required for solutions for injection:

- **Universal Tests**
- **Specific Tests**
  - Antimicrobial Preservatives

**Sterile Powders for Solutions**

Sterile powders for solutions (also referred to as sterile powders for injection) consist of drug substances and other components as dry-formulation ingredients to ensure the chemical and physical stability of the presentation within a final-use container. Companion sterile diluent or diluent compartments may be provided to facilitate constitution to the desired final volume.

The sterile article for injection may be presented in several forms: lyophilized powder intended for final solution, powdered solids intended for final solution, or dry solids that form viscous liquids upon constitution. The description should include a section that deals with ease of dispersion and reconstitution. The dosage form is a homogeneous solid that is readily constituted to the final form with the specified diluent, and dispersion is completed with gentle agitation.

Unless otherwise justified, the following tests apply to sterile powders for injection:

- **Universal Tests**
- The following applies to constituted solutions:
  - Chapter (905): To ensure the consistency of dosage units, each unit in a batch should have a drug substance content within a narrow range around the label claim. Dosage units are defined as dosage forms that contain a single dose or a part of a dose of drug substance in each unit. For liquid dosage forms analysts should conduct the assay on an amount of well-mixed constituted material that is removed from an individual container under conditions of normal use, should express the results as delivered dose, and should calculate the acceptance value.
- The following applies to dry cake:
  - **Loss on Drying** (731): The procedure set forth in this chapter determines the amount of volatile matter of any kind that is driven off under the conditions specified.
  - **Chapter** (921): Water or solvent content may have important effects on reconstitution and stability. For articles that require water or solvent content control, analysts should perform one of the methods described in (921) or a suitable replacement.
  - **Appearance**: Analysts should assess the level of and the unit variation for the following parameters:
    - Color of Dry Cake: Varies within target parameters
    - Texture and Homogeneity of Dry Cake: Varies within target parameters
    - Presence of Foreign Material: All units with visible foreign material must be rejected

**Suspensions**

Parenteral suspensions are liquid dosage forms that contain solid particles in a state of uniform dispersion. Suspensions for parenteral administration must be sterile and compatible with the administration site. Consideration should be given to pH and pyrogenicity, and appropriate limits should be identified. Physical stability evaluations of parenteral suspension preparations should include evaluations to confirm that the particle size range of suspended matter does not change with time and to confirm that the solids in the preparation can be readily resuspended to yield a uniform preparation.

The following tests are required for suspensions for injection unless otherwise justified:

- **Universal Tests**
- **Specific Tests**
  - Uniformity of Dosage Units
  - Antimicrobial Preservatives

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Liposomes

Liposomes are unique drug products with unique properties that can be either solutions or suspensions. Liposomes are aqueous dispersions of amphiphilic lipids and have low water solubility. They are organized as a bilayer sheet that encloses an internal aqueous compartment and are known as lipid bilayer vesicles. Liposomes can have a single lipid bilayer (unilamellar vesicle) or can have an onion-like multilayered structure (multilamellar vesicle). The amphiphilic lipids comprise a hydrated head group at the water interface of the bilayer attached to a hydrophobic group that forms the interior of the bilayer by association with the hydrophobic group of lipids from the opposite leaflet of the bilayer. The physical properties of the liposome and its bilayer can vary widely and depend on lipid composition, aqueous composition, and temperature relative to the acyl components’ phase transition points. Because of the central aqueous compartment, a simple test for the presence of liposomes in a lipid dispersion is to determine the presence of an entrapped aqueous phase.

A liposome drug product consists of the drug substance, liposome components, and other inactive but critical ingredients such as an aqueous dispersion unless the contents are a lyophilized product.

Unless otherwise justified, the following tests are required for liposomes:

- **Universal Tests**
- **Specific Tests**
  - Globule Size Distribution in Lipid Injectable Emulsions (729)

Sterile Powders for Suspensions

Sterile powders for suspensions consist of drug substances and other components as dry-formulation ingredients to ensure the chemical and physical stability of the presentation within a final-use container. Companion sterile diluent or diluent compartments may be provided to facilitate constitution to the desired final volume.

The sterile article for injection may be presented in several forms: lyophilized powder intended for final suspension, powdered solids intended for final suspension, and microparticles that retain their integrity and are delivered as a sterile suspension. The description should include a section that deals with ease of dispersion and reconstitution. The dosage form is a homogeneous solid that is readily constituted to the final form with the specified diluent, and dispersion is completed with gentle agitation.

Unless otherwise justified, the following tests apply to sterile powders for injection:

- **Universal Tests**
- **Microparticles**: Some microparticles are provided as a sterile powder to be reconstituted as a suspension before injection. The majority of microparticle preparations are for reconstitution as a suspension for injection. For quality test requirements, please refer to Implants.

Emulsions

Emulsions for parenteral dosage forms are liquid preparations of drug substances dissolved or dispersed in a suitable emulsion medium. Oil-in-water or water-in-oil emulsions typically entrap the drug substance.

Emulsions typically are white, turbid, homogeneous liquid dosage forms that contain one or more chemical substances (e.g., drug substances and excipients) dissolved in a solvent (aqueous or nonaqueous) or mixture of mutually miscible solvents. Emulsions intended for intravenous administration must be sterile and must be compatible with the intended administration site.

Unless otherwise justified, the following tests are required for emulsions for injection:

- **Universal Tests**
- **Specific Tests**
  - Chapter (729)

Implants

Implants for extended release consist of a matrix of drug substance and polymeric excipient that may or may not have an outer rate-controlling membrane. The polymeric excipient must be biocompatible but may or may not be bioresorbable. Some implants are made from medical-grade metal with an osmotic pump inside that effects the extended release of the drug substance. Implants must be sterile and usually are formed in the shape of a cylinder, although other shapes are used. Solvents used to dissolve the formulation can lead to sterilization, and thus the internal sterility test method should demonstrate that the sample preparation does not lead to sterilization of the test sample.

Cylindrically shaped implants for systemic delivery usually are provided in an inserter for subcutaneous or local administration such as local ocular delivery. Implants also can be surgically implanted for local delivery, e.g., ocular delivery.

Unless otherwise justified, the following tests are required for implants:

- **Universal Tests**
- **Specific Tests**
  - Uniformity of Dosage Units

In situ Gels

Sterile in situ gels are liquid preparations that are intended for injection into specific therapeutic targets. Typically they consist of polymers in organic solvents, and upon injection the solvents migrate away from the site, leaving a gelled mass. The preparations may be injected as-is, upon reconstitution, from in situ formation, or from chemically initiated catalysis that results in the final form.
Unless otherwise justified, the following tests are required for in situ gels:

- **Universal Tests**
- **Specific Tests**
  - Antimicrobial Preservatives

### Microparticles

Injectable, resorbable microparticles for extended release generally range from 20 to 100 µm in diameter. They consist of drug substances embedded within a biocompatible, bioresorbable polymeric excipient, e.g., polyester excipients. Microparticles are provided as a sterile powder in a vial or syringe. Just before intramuscular or subcutaneous administration, the microparticle powder should be suspended in an aqueous injection vehicle (diluent). The injection vehicle usually consists of water for injection, surfactant, and a viscosity enhancer, and the vehicle may contain a compound that adjusts osmolality, e.g., a sugar with or without a compound that controls pH, e.g., an acid. The injection vehicle must be sterile and must be tested according to requirements for solutions that are intended for parenteral administration.

Unless otherwise justified, the following tests are required for microparticles for injection:

- **Universal Tests**
- **Specific Tests**
  - Uniformity of Dosage Units
  - Water Content

### Drug-Eluting Stents

Drug-eluting stents are tiny metal or polymer scaffolds used to keep arteries open following a medical intervention; the drug substance is incorporated into or onto the stent platform. Drug-eluting stents typically have two components of testing: (1) functional tests that generally are American Society for Testing and Materials (ASTM) International methods that fall outside the scope of this chapter and (2) analytical tests.

Unless otherwise justified, the following tests are required for drug-eluting stents:

- **Universal Tests**
- **Specific Tests**
  - Uniformity of Dosage Units
  - Water Content

### LABELING

#### INTRODUCTION

This general chapter provides definitions and standards for labeling of official articles. Labeling standards for an article recognized in USP–NF are expressed in the article’s monograph and applicable general chapters. It is intended that all articles in USP or NF will be subject to the labeling requirements specified in this chapter by means of a provision in General Notices, 10 Preservation, Packaging, Storage, and Labeling, unless different requirements are provided in a specific monograph. As with compendial standards for naming, identity, strength, quality, and purity, compendial requirements for labeling have a role in the adulteration and misbranding provisions of federal law (see the Federal Food, Drug, and Cosmetic Act (FDCA) sections 501(b), 502(e)(3)(b), 502(g), and 502(h)). Exceptions or additional requirements specific to animal drug products and compounded preparations are provided in separate sections. Vaccine labeling is not included in this general chapter.

#### DEFINITIONS

The term “labeling” includes all labels and other written, printed, or graphic matter on an article’s immediate container or on, or in, any package or wrapper in which it is enclosed, except any outer shipping container. The term “label” is that part of the labeling on the immediate container.

A shipping container that contains a single article, unless the container also is essentially the immediate container or the outside of the consumer package, must be labeled with a minimum of product identification (except for controlled substances), lot number, expiration date, and conditions for storage and distribution.

Beyond-use dates (BUDs) and expiration dates are not the same. An expiration date identifies the time during which a conventionally manufactured product, active ingredient, or excipient can be expected to meet the requirements of a compendial monograph, if one exists, provided it is kept under the prescribed storage conditions. The expiration date limits the time during which the conventionally manufactured product, active pharmaceutical ingredient (API), or excipient may be dispensed or used. Expiration dates are assigned by manufacturers of conventionally manufactured products based on analytical
and performance testing of the sterility, chemical and physical stability, and packaging integrity of the product. Expiration dates are specific for a particular formulation in its container and at stated exposure conditions of illumination and temperature. The beyond-use date (BUD) is the date or time beyond which a compounded preparation must be discarded. The date or time is determined from the date the preparation was compounded.

LABELS AND LABELING FOR DRUG PRODUCTS AND COMPOUNDED PREPARATIONS EXPRESSED AS ACTIVE MOIETY IN NAME AND STRENGTH

The names and strengths of drug products and compounded preparations formulated with a salt of an acid or base are to be expressed in terms of the active moiety on the label (see Nomenclature (1121), Monograph Naming Policy for Salt Drug Substances in Drug Products and Compounded Preparations).

Labeling

The labeling clearly states the specific salt form of the active moiety that is present in the product or preparation because this information may be useful to practitioners and patients. The names and strengths of both the active moiety and specific salt form (when applicable) are provided in the labeling.

Exceptions

In rare cases in which the use of the specific salt form of the active moiety in the title provides vital information from a clinical perspective, an exception to this policy may be considered. In such cases, when the monograph title contains the specific salt form of the active moiety, the strength of the product or preparation is also expressed in terms of the specific salt form.

LABELS AND LABELING FOR INJECTABLE PRODUCTS

The labels1 and the labeling state the following information:

- Name of the product
  - In the case of a liquid, the quantity or proportion of each active moiety or drug substance in a specified volume
  - In the case of any product to which a diluent must be added before use, the quantity or proportion of each active moiety or drug substance, name and volume of diluent to be added, the concentration after the diluent is added, directions for proper storage of the constituted solution, and a BUD (see Expiration Date and Beyond-Use Date).
- Route(s) of administration
- Name and quantity or proportion of all inactive ingredients except ingredients added to adjust the pH or to make the drug isotonic may be declared by name with a statement of their effect; if the vehicle is Water for Injection it need not be named.
- Statement of storage conditions
- Name and place of business of the manufacturer, packer, or distributor
- Identifying lot number and expiration date
- “Rx only” for human drugs
- The recommended or usual dosage.

The container must be labeled so that a sufficient area of the container remains uncovered for its full length or circumference to permit inspection of the contents.

The lot number must be traceable to the complete manufacturing history of the specific package, including all manufacturing, filling, sterilizing, and labeling operations.

If the individual monograph permits varying concentrations of active moiety or drug substance in a large-volume injection (LVI), the concentration of each active moiety or drug substance named in the official title is stated as if it were part of the official title (e.g., 5% Dextrose Injection, or Dextrose Injection 5%, or 5% Dextrose and 0.2% Sodium Chloride Injection or Dextrose (5%) and Sodium Chloride (0.2%) Injection).

Quantity and Total Volume for Injectable Drug Products Packaged in Single- and Multiple-Dose Containers

For injectable drug products greater than 1 mL, whether packaged in single- or multiple-dose containers, the quantity per total volume should be the primary and prominent expression on the principal display panel of the label, followed in close proximity by quantity per milliliter enclosed by parentheses (quantity/mL).

For containers that hold a volume of less than 1 mL, the quantity per fraction of a milliliter should be the only expression of strength. For containers that hold a volume equal to 1 mL, the strength should be expressed as quantity per milliliter (quantity/mL), not quantity/1 mL.

The following example formats are acceptable:

1. For containers less than 1 mL: 12.5 mg/0.625 mL
2. For containers equal to 1 mL: 5 mg/mL (not 5 mg/1 mL)

1 If there are space limitations, see 21 CFR§ 201.10(i), 21 CFR§ 201.105(b), 21 CFR§ 610.60.
3. For containers greater than 1 mL:

Example 1: 500 mg/10 mL
   (50 mg/mL)

Example 2: 25,000 Units/5 mL
   (5,000 Units/mL)

In certain cases, the primary and prominent expression of the total drug content per container is not effective in preventing medication errors and therefore in those cases, the total drug content per container should not be the primary and prominent expression of strength. Insulin products are an example of a product class that is an exception from the total drug content per container requirement. Another exception to expressing strength as quantity per total volume is lidocaine (or similar drugs for local anesthesia) where the product may be ordered and administered by percentage (e.g., 1% or 2%). In such cases, the percentage strength as well as the quantity per total volume followed in close proximity by quantity per milliliter enclosed by parentheses must be used.

Example 1: 1%
   (100 mg/10 mL)
   (10 mg/mL)

Example 2: 2%
   (1000 mg/50 mL)
   (20 mg/mL)

Dry solids that must be constituted should follow the same format with the exception that only the quantity of the drug in the container should be listed as the primary expression of strength, not the quantity per total volume or quantity per milliliter (quantity/mL).

Example: 500 mg/vial

**Ratio Expression of Strength**

Single-entity injectable drug products must be labeled in terms of quantity per milliliter (quantity/mL) and not as a ratio expression.

Examples:
- Epinephrine Injection, 1:1000 must be expressed as 1 mg/mL.
- Epinephrine Injection, 1:10,000 must be expressed as 0.1 mg/mL.
- Isoproterenol Hydrochloride Injection, 1:5000 must be expressed as 0.2 mg/mL.
- Neostigmine Methylsulfate Injection, 1:1000 must be expressed as 1 mg/mL.

Single-entity injectable drug products greater than 1 mL should be formatted as quantity per total volume on the principal display panel of the label followed in close proximity by quantity per milliliter (quantity/mL) enclosed by parentheses. When combined with a local anesthetic, the concentration of epinephrine will be expressed as a ratio.

Examples:
- Lidocaine Hydrochloride and Epinephrine Injection 1%/1:100,000
  
  or

- Lidocaine Hydrochloride 1%
  
  and

- Epinephrine Injection 1:100,000

**Pharmacy Bulk Package**

Where a container is offered as a Pharmacy Bulk Package, the label must: 1) state prominently “Pharmacy Bulk Package—Not for Direct Infusion”; 2) contain or refer to information on proper techniques to help assure safe use of the product; and 3) bear a statement limiting the time frame in which the container may be used once it has been entered, provided it is held under labeled storage conditions (see Packaging and Storage Requirements (659)).

**Imaging Bulk Package**

Where a container is offered as an Imaging Bulk Package, the label must: 1) state prominently “Imaging Bulk Package” and, in juxtaposition with this statement, include the following use statement: “For use only with an automated contrast injection system, contrast management system, or contrast media transfer set approved or cleared for use with this contrast agent in this Imaging Bulk Package”; 2) bear a statement limiting the time frame in which the container may be used once it has been entered, provided it is held under the labeled storage conditions; 3) bear the statement “See drug and device labeling for information on devices indicated for use with this Imaging Bulk Package and techniques to help assure safe use.” (See (659).)
Ferrules and Cap Overseals

Healthcare practitioners using injectable products must be able to easily see and act on labeling statements that convey important safety messages critical for the prevention of imminent life-threatening situations. These cautionary labeling statements must be simple, concise, and devoid of nonessential information. Products that do not require cautionary statements should be free of information, so that those with cautionary statements are immediately apparent. Accomplishing this requires a systematic approach to the labeling of injectable products, and one that ensures that the ferrule and cap overseal—an area of these products that is highly visible to practitioners as they use these medicines—is reserved for critical safety messages. Accordingly:

1. Only cautionary statements may appear on the top (circle) surface of the ferrule and cap overseal of a vial containing an injectable product. The cautionary statement should appear on both the ferrule and cap, but may appear solely on the ferrule if the cap overseal is transparent and the cautionary statement beneath the cap is readable. A cautionary statement is one intended to prevent an imminent life-threatening situation and may include instructional statements that provide potency or other safety-related instructions if warranted. Examples of such statements include, but are not limited to: “Warning—Paralyzing Agent” and “Dilute before Using.” The cautionary statement should be printed in a contrasting color and should be clearly visible under ordinary conditions of use.

2. If no cautionary statement is necessary, the top surface of the vial, including the ferrule and cap overseal, must remain blank.

3. Other statements or features including, but not limited to, identifying numbers or letters, such as code numbers, lot numbers, company names, logos, or product names, etc., may appear on the side (skirt) surface of the ferrule on vials containing injectable products, but not on the top (circle) surface of the ferrule or cap overseal. The appearance of such statements or features on the skirt surface of the ferrule should not detract from, or interfere with, the cautionary statement on the top surface.

Potassium Chloride for Injection Concentrate

The use of a black closure system on a vial (e.g., a black cap overseal and a black ferrule to hold the elastomeric closure) or the use of a black band or series of bands above the constriction on an ampule is prohibited, except for Potassium Chloride for Injection Concentrate (see (659)).

Neuromuscular Blocking and Paralyzing Agents

All injectable neuromuscular blocking agents and paralyzing agents must be packaged in vials with a cautionary statement printed on the ferrules and cap overseas. Both the container cap ferrule and the cap overseal must bear in black or white print (whichever provides the greatest color contrast with the ferrule or cap color) the words: “Warning: Paralyzing Agent” or “Paralyzing Agent” (depending on the size of the closure system). Alternatively, the overseal may be transparent and without words, allowing for visualization of the warning labeling on the closure ferrule.

Aluminum in Large-Volume Injections (LVIs), Small-Volume Injections (SVIs), and Pharmacy Bulk Packages (PBPs) Used in Parenteral Nutrition (PN) Therapy^2

1. The aluminum content of LVIs used in PN therapy must not exceed 25 mcg/L.
2. The package insert of LVIs used in PN therapy must state that the drug product contains no more than 25 mcg of aluminum per liter. This information must be contained in the Precautions section of the labeling of all LVIs used in PN therapy.
3. If the maximum amount of aluminum in SVIs and PBPs is 25 mcg/L or less, instead of stating the exact amount of aluminum that each contains, as in paragraph (4), the immediate container label for SVIs and PBPs used in the preparation of PN admixtures or formulations (with exceptions as noted below) may state: “Contains no more than 25 mcg/L of aluminum.” If the SVI or PBP is a lyophilized powder, the immediate container label may state the following: “When reconstituted in accordance with the package insert instructions, the concentration of aluminum will be no more than 25 mcg/L.”
4. The maximum level of aluminum at expiry must be stated on the immediate container label of all SVIs and PBPs used in the preparation of PN admixtures or formulations. The aluminum content must be stated as follows: “Contains no more than ___ mcg/L of aluminum.” The immediate container label of all SVIs and PBPs that are lyophilized powders used in the preparation of PN solutions must contain the following statement: “When reconstituted in accordance with the package insert instructions, the concentration of aluminum will be no more than ___ mcg/L.” This maximum amount of aluminum must be stated as the highest one of the following three levels:
   • The highest level for the batches produced during the past 3 years
   • The highest level for the latest 5 batches
   • The maximum level in terms of historical levels, but only until completion of production of the first 5 batches.

   The package insert for all LVIs, SVIs, and PBPs used in the preparation of PN admixtures or formulations must contain the following statement in the Warnings section of the labeling:

^2 See 21 CFR §201.323. USP uses the following terms: large-volume injections (LVIs), small-volume injections (SVIs), and parenteral nutrition (PN), rather than terminology used in 21 CFR §201.323: large-volume parenterals, small-volume parenterals, and total parenteral nutrition.
LABELS AND LABELING FOR PRODUCTS IN OTHER CATEGORIES

Labels and Labeling of Liquids and Constituted Products

The labels’ and labeling state the following information:

1. In the case of a liquid product, the percentage content of each active moiety or drug substance or the amount of each active moiety or drug substance in a specified volume.

2. In the case of a product to which a diluent must be added before use, the amount of each active moiety or drug substance, the name and volume of diluent to be added, the final volume of solution, the concentration after the diluent is added (e.g., quantity/mL or quantity/5 mL), directions for proper storage of the constituted solution, and an expiration or BUD (see Expiration Date and Beyond-Use Date).

Amount of Active Moiety or Drug Substance per Dosage Unit

The strength of a drug product is expressed on the container label in terms of micrograms, milligrams, grams, or percentage of the therapeutically active moiety or drug substance, whichever form is used in the title, unless otherwise indicated in an individual monograph. Both the active moiety and drug substance names and their equivalent amounts are then provided on the container label and in the labeling (see Nomenclature (1121); Monograph Naming Policy for Salt Drug Substances in Drug Products and Compounded Preparations).

Official articles in capsule, tablet, or other dosage forms must be labeled to express the quantity of each active moiety or drug substance contained in each unit. Unit-dose oral solutions or suspensions (whether supplied as liquid products or as liquid products that are constituted from solids upon addition of a designated volume of a specific diluent) must be labeled to express the quantity of each active moiety or drug substance delivered under the conditions prescribed in Deliverable Volume (698).

Official drug products not in unit-dose packaging must be labeled to show the quantity of each active moiety or drug substance in each milliliter or in each gram, or to express the percentage of each such ingredient (see General Notices, 8.140 Percentage Concentrations). Exceptions are oral liquids or solids intended to be constituted to yield oral liquids that, alternatively, can be labeled in terms of each 5-mL portion of the liquid or resulting liquid. Unless otherwise required by regulation [e.g., over-the-counter (OTC) regulation 21 CFR §201.62(b)] or indicated in a monograph or chapter, declarations of strength or quantity must be stated only in metric units [see also General Notices, 5.50.10 Units of Potency (Biological)].

Expiration Date and Beyond-Use Date

The label of an official drug product or nutritional or dietary supplement product shall bear an expiration date. All products shall display the expiration date so that it can be read by an ordinary individual under customary conditions of purchase and use. The expiration date shall be prominently displayed in high contrast to the background or it shall be sharply embossed, and easily understood (e.g., “EXP 6/13,” “Exp. June 13,” or “Expires 6/2013”).

[Note—For additional information and guidance, refer to the Consumer Products Association’s Voluntary Codes and Guidelines of the Consumer Healthcare Products Industry.] The monographs for some preparations state how the labeled expiration date shall be determined. In the absence of a specific requirement in the individual monograph for a drug product or nutritional supplement, the label shall bear an expiration date assigned for the particular formulation and package of the product, with the following exceptions: the label need not show an expiration date if the drug product or nutritional supplement is packaged in a container that is intended for sale without prescription, and the labeling states no dosage limitations, and if the product or supplement is stable for NLT 3 years when stored under the prescribed conditions.

If an official product is required to bear an expiration date, the product shall be dispensed solely in or from a container labeled with an expiration date, and the date on which the article is dispensed shall be within the labeled expiry period. The expiration date identifies the time during which the article can be expected to meet the requirements of the compendial monograph, provided it is kept under the prescribed storage conditions. The expiration date limits the time during which the article may be dispensed or used. If an expiration date is stated only in terms of the month and the year, then the intended expiration date is the last day of the stated month.

The beyond-use date is the date after which a product shall not be used. The dispenser shall place on the label of the prescription container a suitable beyond-use date to limit the patient’s use of the article based on any information supplied by the manufacturer or this subsection. The beyond-use date shall not be later than the expiration date on the manufacturer’s container. Also see the section Compounded Preparations below.

For articles that require constitution before use, a suitable beyond-use date for the constituted product shall be identified in the labeling.

For all other dosage forms, in determining a beyond-use date the dispenser shall take into account, in addition to any other relevant factors:
Labels and Labeling for Compounded Preparations

The labels and labeling state the following information:

In the case of a compounded preparation, list the name(s) and amount(s) or concentrations of active moiety(ies) or drug substance(s) on the immediate container (see Pharmaceutical Compounding—Sterile Preparations (797), Labeling (CN 1-May-2020) and Pharmaceutical Compounding—Nonsterile Preparations (795), Labeling (CN 1-May-2020)).

In the case of a compounded preparation prepared in 503A facilities as defined by FDCA §503A, the labeling should indicate that “this is a compounded preparation.”

The label on the container or package of a compounded preparation must bear a BUD. The BUD is determined from the date the preparation is compounded. Because compounded preparations are intended for administration immediately or following short-term storage, their BUDs may be assigned based on criteria different from those applied to assigning expiration dates to manufactured drug products.

The monograph for an official compounded preparation typically includes a BUD that states the time period following the date of compounding during which the preparation, properly stored, may be used. For guidance regarding the BUD for compounded sterile and nonsterile preparations, see 797 and 795, respectively.

The label on the official compounded preparation must include the word “compounded” after the drug portion of a non-proprietary name (e.g., “Baclofen Compounded Oral Suspension”).

Dialysis, Hemofiltration, and Irrigation

Solutions that are intended for use as dialysis, hemofiltration, or irrigation, and are packaged in a container with a volume of more than 1 L, must be labeled to indicate that the contents are not to be administered either intravenously or intra-arterially.

Use of Leading and Terminal Zeros

To help minimize the possibility of errors in drug dispensing and administration, when the quantity of active moiety or drug substance is expressed in whole numbers it must be shown without a decimal point followed by a terminal zero (e.g., express as 4 mg, not 4.0 mg). When the quantity of active moiety or drug substance is expressed as a decimal number smaller than 1, it must be shown with a zero preceding the decimal point (e.g., express as 0.2 mg, not .2 mg).

Units

Abbreviations for the terms “Units” or “International Units” must not be used for labeling or prescribing purposes. Examples include “U”, “u”, and “IU”. Medication errors have occurred when these abbreviations have been used. See General Notices, 9.10 Use of Metric Units.

Alcohol

The alcohol content in a liquid formulation must be stated on the label as a percentage (v/v) of alcohol (C₂H₅OH).

Botanicals

The label of an herb or other botanical intended for use as a dietary supplement shall bear the statement, “If you are pregnant or nursing a baby, seek the advice of a health professional before using this product.”

Electrolytes

The concentration of each electrolyte for replacement therapy (e.g., sodium, potassium, chloride) must be stated on the label in milliequivalents per volume (mEq/volume). Phosphorus containing injections must be expressed in milliMoles per volume (e.g., mM/volume). The label of the product must also indicate the quantity of ingredient(s) in terms of weight or percentage concentration.
Non-Oral Products

Non-oral product labels and labeling must state the names of added substances (as defined in General Notices, 5.20 Added Substances) in compliance with 21 CFR §201.100(b)(5).

Salts of Drugs

It is an established principle that official articles must have only one official title (see General Notices, 2.20 Official Articles and compendial nomenclature requirements in (1121)). For purposes of saving space on labels and because chemical symbols for the most common inorganic salts of drugs are well known to practitioners, the following alternatives are permitted in labeling official articles that are salts: HCl for hydrochloride; HBr for hydrobromide; Na for sodium; and K for potassium. The symbols Na and K are intended for use in abbreviating names of the salts of organic acids, but these symbols are not used when the word Sodium or Potassium appears at the beginning of an official title (e.g., Phenobarbital Na is acceptable, but Na Salicylate is not).

Special Capsules and Tablets

The label of any form of capsule or tablet intended for administration other than by swallowing intact must bear a prominent indication of the manner in which it should be used (see Compendial Nomenclature, Nomenclature Guidelines on the USP website at www.usp.org).

Products That Contain Vitamins

The vitamin content of a drug product must be stated on the label in metric units per dosage unit. The amounts of vitamins A, D, and E may also be stated in USP Units. Quantities of vitamin A declared in metric units refer to the equivalent amounts of retinol (vitamin A alcohol).

Controlled Room Temperature

Articles may be labeled for storage at “controlled room temperature” or at “20° to 25°”, or other equivalent wording based on the same mean kinetic temperature. All three labeling options must ensure not to exceed the mean kinetic temperature of 25° with excursions between 15° and 30° (see (659)).

Light-Resistant Container

When an opaque covering is used to provide protection from light for a light-sensitive product packaged in a clear or colorless or translucent container, the label of the container bears a statement that the opaque covering is needed until the contents are to be used or administered (see Containers—Performance Testing (671), Spectral Transmission and (659)).

Single-Unit Container

Each single-unit container must be labeled to indicate the identity; quantity and/or strength; name of the manufacturer, packer, or distributor; lot number; and expiration date of the article (see (659)).

Single-Dose Container

When space permits a single-dose container must be labeled as such, and should include on the label appropriate discard instructions (see (659)).

Multiple-Dose Container

When space permits, a multiple-dose container must be labeled as such (see (659)).

Unit-of-Use Container

A unit-of-use container must be labeled as such (see (659)).

Protection from Freezing

The container label must bear an appropriate instruction to protect the article from freezing if subject to loss of strength or potency, or to destructive alteration of its characteristics (see (659)).
Prescription Container Labeling

At a minimum, a prescription container must be labeled in a patient-centered manner. The label must contain essential information that is important for the patient’s safe and effective use of the medicine. Labels should be designed and formatted to optimize readability and understanding (see Prescription Container Labeling (17)).

GENERAL LABELING

Users are reminded to always refer to the General Notices in assessing or applying any compendial standards. General Notices addresses a number of labeling-related aspects, including 3.20 Indicating Conformance (when an article may be labeled USP, NF, or USP–NF, and requirements related to differences in identity, naming, strength, quality, or purity); General Notices, 5.20.20 Added Substances (Excipients and Ingredients) in Official Products; 6.70 Reagents; and 8.240 Weights and Measures (e.g., microgram may be represented as either µg or mcg. For labeling or prescribing purposes, only “mcg” is to be used).

LABELS AND LABELING FOR ANIMAL DRUG PRODUCTS

This section provides exceptions or additions to the previous requirements in this chapter. The following requirements are specific to the labeling for animal drug products.

Definitions

The written printed or graphic matter on the outer shipping container for animal drugs is considered to be part of “labeling”. Shipping labeling for animal drugs should contain, at minimum, product identification, lot number, expiration date, and conditions for storage and handling.

Labeling for Animal Drug Products

The labeling for animal drugs should identify the animal species and, if applicable, specific subset(s) of the animal species for which the drug is approved, conditionally approved, or indexed. In the case of a compounded preparation for animals, the labeling should indicate that “This is a compounded preparation.”

The labeling for prescription animal drug products must include the following statement:
• "Caution: Federal law restricts this drug to use by or on the order of a licensed veterinarian."

Labeling for Injectable Animal Drug Products

Labeling for injectable animal drug products includes the following information:
• A statement limiting the time frame in which the container may be used once it has been entered (e.g., needle-punctured), provided it is held under the labeled storage conditions.

Quantity and Total Volume for Single- and Multiple-Dose Injectable Animal Drug Products

Because of considerable variability in body weight within and among animal species, most injectable animal drugs are approved to be dosed on a mg/kg body weight basis. Thus, strength on labeling for single- and multiple-dose injectable animal drugs should be expressed on the basis of quantity per milliliter (quantity/mL), usually mg/mL. An exception would be for single-dose injectable animal drugs that are dosed regardless of the weight of the animal, in which case strength on labeling should be expressed as quantity per total volume (e.g., 50 mg/5 mL). For single-dose containers holding less than 1 mL, strength on labeling should be expressed as quantity per fraction of a mL (e.g., 12.5 mg/0.625 mL).

Dry solids that must be constituted should provide on labeling the total quantity followed by the quantity per milliliter (quantity/mL) after constitution.

Compounded Veterinary Preparations

The label on the official compounded veterinary preparation must include the word “compounded” after the drug portion of a non-proprietary name and the word “veterinary” at the end of the full official name (e.g., Atenolol Compounded Oral Suspension, Veterinary).
USP REFERENCE STANDARDS FOR USP OR NF

Official applications of USP RS are specified in USP or NF monographs and general chapters. These applications are as follows. Some USP RS could be used for several types of applications listed below.

1. Quantitative determinations
   A. The majority of USP RS for quantitative determinations support measurements for total amounts of material on a mass basis. This category includes USP RS for USP or NF articles and impurity standards labeled for quantitative use. The assigned value of the USP RS is stated on the labeling and should be included in calculations used in the monograph and applicable general chapters.
   B. USP RS for relative determinations of potency or activity are often required for the measurement of complex materials (e.g., biologics, antibiotics, herals, some dietary supplements) and quantitative amounts may be expressed in units or relative potency terms other than mass. These USP RS are established by calibration to a primary standard where the property of the material determines the unit. For these standards where an International Standard (IS) established by the WHO exist, USP RS are National Measurement Standards. The USP RS documentation will indicate when the USP RS has been established by comparison to an International Standard (IS) established by the WHO. Results may be expressed in USP Units, Units, or International Units. Additional statements about unit/mass relationship, specific activity, or other relevant information related to the measurement may be provided in the USP RS documentation.
   For antibiotics that use microbial assays to determine activity, the potency is determined in units or micrograms per milligram (µg/mg) of activity. The units or µg/mg of activity is established against a WHO IS when one exists for that antibiotic. Where no WHO IS is presently available, USP establishes and maintains the standard to which USP RS lots are calibrated. This approach may also be chosen for other complex materials for which no WHO IS exists. In these instances, the USP standard is established in such a way as to ensure long-term stability and fitness for purpose, which permits the calibration of successive lots of USP RS with increased confidence that drift in the assigned unit can be avoided.

2. Qualitative determinations
A. Identification USP RS: USP RS for identification tests are typically presented as single components of high chemical purity, but may also be complex materials of natural, synthetic, or recombinant origin (e.g., biologics, natural products, botanicals, complex nonbiologics, others). For complex materials, the identification attributes are presented in a matrix of other materials and require highly specific measurement systems (e.g., nucleic acid-based identity determination for naturally derived materials).

B. Impurity USP RS: Impurity USP RS are typically used for system suitability or as impurity markers. They may be presented as single-component materials, as mixtures containing more than one impurity, or as drug substance(s) containing one or more impurities.

C. Digital and Visual USP RS: Unlike chemical reference materials, these USP RS are not physical materials used in chemical analyses. Instead, these visual images are used by analysts to compare test articles to ensure that they meet compendial requirements

3. Performance verification. These USP RS are typically called for in general tests and assays and are provided to analyze and, where appropriate, to facilitate adjustment of the operation of an instrument to ensure the results obtained are accurate and/or precise or otherwise give acceptable results. The use of these USP RS is generally described in associated general chapters and in the supporting documentation supplied with the USP RS.

**USP REFERENCE STANDARDS FOR OTHER MEASUREMENTS AND DETERMINATIONS**

USP also develops Reference Standards that may not be required in official USP–NF tests or assays. USP provides RS specified in the current edition of the Food Chemicals Codex, the Herbal Medicines Compendium, and standards referenced in regulatory requirements.

USP RS without an official use in the USP–NF are developed following the same quality systems used for the characterization and release of USP RS used in official tests and assays. These USP RS are generally intended to address common quality issues and challenges inherent to technologies that cut across different types of products (e.g., system suitability samples, calibrators used to demonstrate performance of an analytical procedure, process, or equipment). Extensive characterization of the USP RS candidate is required and the testing plan takes into account the use of different methods to measure the same attribute, demonstrating broader applicability of these standards. In the absence of a companion monograph or chapter, the information generated from these studies may be disseminated to the user via other types of supporting documents including but not limited to the USP Certificate.

**LABELING**

The labeling material consists of the label affixed to the USP RS and the associated USP Certificate. Both must be reviewed prior to handling or using the USP RS because in some cases not all of the necessary information can fit on the affixed label. USP Certificates are lot specific and are publicly available on the USP website (www.usp.org). Additional documentation may be provided with the USP RS as needed.

The affixed USP RS label typically contains the RS name, catalog number, lot number, package size, assigned value, storage conditions, handling instructions, and country of origin information. For multi-component items, there is also an outer package and label.

The affixed label also includes hazard and precautionary statements required by the Occupational Safety and Health Administration (OSHA) under the current revision of the Hazard Communication Standard (29 CFR 1910.1200). Terms used in these statements do not necessarily reflect specific definitions in the USP–NF. Safety Data Sheets for all USP RS are publicly available on the USP website (www.usp.org).

In addition to the information provided on the affixed USP RS label, the USP Certificate will generally contain the RS chemical name and structure, sequence (if applicable), CAS number, molecular formula, and molecular weight. A typical chromatogram may also be included if necessary for the intended use. Additional information may be included such as special handling instructions or information needed for the use of the USP RS. The USP Certificate also includes a copy of the label text and a series of general instructions.

**PACKAGING**

The amount of material per individual USP RS container depends on the application of the standard. Some standards (mainly materials with significant handling requirements or materials that are available only in small amounts) are provided in single-use containers. Some single-use products may be lyophilized with content labeled in mass or activity units per container. If so labeled, the content of the container must be reconstituted in its entirety without any additional weighing. Instructions for use are given either on the label or USP Certificate, or in the monographs where the standard is used.

**STORAGE**

USP RS should be stored in the packaging configuration provided by USP, according to the label and USP Certificate instructions. When storage in refrigerator or freezer is stated on the label, follow the definitions given in Packaging and Storage Requirements (659). If no specific directions or limitations are provided on the label, the conditions of storage shall be room temperature and protection from moisture, light, freezing, and excessive heat.

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Any unused portions remaining after the container has been opened should be carefully stored in accordance with the user’s Standard Operating Procedures and good laboratory practices. Decisions concerning the proper use of previously opened USP RS are the responsibility of the user, unless otherwise specified on the labeling. The user is responsible for ensuring that the contents of opened vials continue to be suitable for their intended use.

CONTINUED SUITABILITY FOR USE

All USP RS are periodically reevaluated by USP throughout their lifecycles. The USP Continued Suitability for Use (CSU) program is designed to monitor real-time suitability for use of all current lots of USP RS. Suitability testing intervals are established based on collaborative study data, manufacturer or supplier data, testing results, and CSU data trending and projections. When and where applicable, an accelerated degradation study may be performed to provide additional information on the stability of the USP RS and to support suitability testing intervals. The goal of the CSU program is to confirm the continued suitability of the material for use of a USP RS in its compendial applications during its valid use period.

VALID USE DATE

USP RS lots are assigned a valid use date upon depletion. The valid use date is the last day upon which a particular lot of USP RS can be used. Typically, the valid use date assigned is one year from the date the last vial of a lot is sold.

It is the responsibility of the user to ascertain that a particular lot of a USP RS has official status either as a “Current Lot” or as a “Previous Lot” prior to the valid use date. Current and previous lot information, as well as the most current version of the catalog, can be found on the USP website (www.usp.org).

PROPER USE

Many compendial tests and assays are based on comparison of a sample to a USP RS. In such cases, measurements are made on preparations of both the sample and the USP RS. Where it is directed that a standard solution or a standard preparation be prepared for a quantitative determination, it is intended that the USP RS substance be accurately weighed (see Balances (41)) and subsequent dilutions be performed using volumetric apparatuses with, at least, the prescribed tolerances (see Volumetric Apparatus (31)). Potential errors associated with the use of volumetric apparatus of small volume should be taken into account (see also General Notices, 6.50.20.1 Adjustments to Solutions).

Whenever the labeled directions for use require either drying or a correction for water and/or volatiles, this should be performed at the time of use. Further experimental details should be controlled by the user’s Standard Operating Procedures and good laboratory practices.

The following list of label terms and definitions is provided as guidance for the handling and use of USP RS:

- **Assigned Value (Calculation Value):** The quantity value assigned to a USP RS for its use in the quantitative compendial applications.
- **As Is:** Use the USP RS as received, without drying or additional testing and apply the assigned value to correct the concentration of the standard solution and/or preparation. This is the preferred option, and is selected whenever data indicate the moisture content is constant over time. For the USP RS to be used on the as is basis, the assigned value has already been corrected for volatiles, including moisture.
- **Anhydrous Basis, Determine Water Content Titrimetrically at Time of Use:** Use the USP RS as received, and apply the water content determined to correct the weight of the standard. After the correction for water is applied, use the assigned value to correct the concentration of the standard solution and/or preparation. At the time a USP RS is to be weighed, proceed as directed under Water Determination (921), Method I, and determine the water content on a separate portion of material. Instrumental or microanalytical methods are acceptable for this purpose. When using typical amounts (about 50 mg of the USP RS), titrate with a 2- to 5-fold dilution of the reagent.
- **Dried Basis, Determine Loss on Drying at Time of Use:** Use the USP RS as received, and apply the loss on drying value obtained to correct the weight of the standard. After the correction for loss on drying is applied, use the assigned value to correct the concentration of the standard solution and/or preparation. Determine the loss on drying value on a separate portion of material, following the monograph procedure under Loss on Drying. Sample sizes smaller than those required in the general test chapter may be used for a USP RS provided that the user can obtain a sufficiently accurate result.
- **Dried Material, Dry Before Use, or Use Previously Dried Material:** Dry the USP RS before use. Use immediately after drying under stated conditions. Drying should not be performed in the original container. A portion of the material should be transferred to a separate drying vessel. Apply the assigned value to correct the concentration of the standard solution and/or preparation prepared with the dried USP RS.▲ (USP 1-Aug-2020)

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INTRODUCTION

Antimicrobial preservatives are substances added to aqueous pharmaceutical products. Nonsterile dosage forms may have preservatives added to protect them from growth of microorganisms inadvertently introduced during or subsequent to the manufacturing process. In the case of sterile articles packaged in multiple-dose containers, antimicrobial preservatives are added to inhibit the growth of microorganisms that may be introduced from repeatedly withdrawing individual doses. One or more antimicrobial preservative(s) are expected in all sterile multidose units.

Antimicrobial preservatives should not be used as a substitute for good manufacturing practices, solely to reduce the viable microbial population of a nonsterile product, or control the presterilization bioburden of a multidose formulation during manufacturing. Antimicrobial preservatives in compendial dosage forms meet the requirements for General Notices, 5.20 Added Substances.

All useful antimicrobial agents are toxic substances. For maximum protection of patients, the concentration of the preservative shown to be effective in the final packaged product should be below a level that may be toxic to human beings based on the recommended dosage of the medicinal product.

The concentration of an added antimicrobial preservative can be kept to a minimum if the active ingredients of the formulation possess an intrinsic antimicrobial activity. Antimicrobial effectiveness, whether inherent in the product or produced because of the addition of an antimicrobial preservative, must be demonstrated for all injections packaged in multiple-dose containers or for other products containing antimicrobial preservatives. Antimicrobial effectiveness must be demonstrated for aqueous-based, multiple-dose topical and oral dosage forms and for other dosage forms such as ophthalmic, otic, nasal, irrigation, and dialysis fluids (see Pharmaceutical Dosage Forms (1151)). For the purpose of the test, aqueous is defined as a water activity of more than 0.6 (see Application of Water Activity Determination to Nonsterile Pharmaceutical Products (1112)).

Challenge organisms are generally based on likely contaminants to a drug product while considering its physical attributes, formulation, and intended use. The standard battery of challenge organisms described in this test need not prevent the inclusion of other species of microorganisms if deemed useful to measure the biological activity of the preservative system for a specific product. These supplemental challenge organisms are not within the scope of this chapter, but may be added in addition to the described test organisms.

GENERAL PROCEDURES

This chapter provides procedures to demonstrate the effectiveness of added antimicrobial preservatives. Such antimicrobial preservatives must be declared on the label. The procedures and acceptance criteria for effectiveness apply to a product in the original, sealed container in which it was distributed by the manufacturer (see Table 1 for categories of products). The test need not be conducted in these containers, but care should be taken to avoid using materials that can interact with the preservative in the containers that are used for antimicrobial effectiveness testing.

Growth Promotion Procedure and Suitability of the Recovery Method

GENERAL CONSIDERATIONS

The ability of the procedure to detect challenge microorganisms in the presence of a suitably neutralized product to be tested must be established. The suitability of the procedure must be reconfirmed if a change is made in materials or methods or if a change is made in the product or direct product contact materials that may affect the outcome of the test.

The growth-promoting capabilities of media used in this procedure must be established.

PREPARATION OF TEST STRAINS

Use standardized suspensions of test strains or prepare as stated below. Seed-lot culture maintenance techniques (seed-lot systems) are used so that the viable microorganisms used for inoculation are NMT five passages removed from the original master seed lot. Grow each of the bacterial and fungal test strains separately (see Table 2).

Use cultures of the following microorganisms:1 Candida albicans (ATCC No. 10231), Aspergillus brasiliensis (ATCC No. 16404), Escherichia coli (ATCC No. 8739), Pseudomonas aeruginosa (ATCC No. 9027), and Staphylococcus aureus (ATCC No. 6538). The viable microorganisms used in the procedure should be part of a freshly growing culture (e.g., in logarithmic growth phase) with the exception of A. brasiliensis spores. The culture conditions for the inoculum culture are described (see Table 2) in which the suitable media are Soybean–Casein Digest broth or Sabouraud Dextrose Agar Medium.

To harvest the bacterial and C. albicans cultures, use sterile saline TS to wash the surface growth, and collect it in a suitable vessel. To harvest the spores of A. brasiliensis, use sterile saline TS containing 0.05% of polysorbate 80. The spore suspension should be aseptically treated (e.g., filtration through sterile glass wool) to remove hyphae. All microbial suspensions should be prepared to ensure that there is no carry over of residual growth medium from the inoculum (e.g., centrifugation followed by resuspension in appropriate sterile suspending fluid.)

Alternatively, the stock culture organisms may be grown in a suitable liquid medium (i.e., Soybean–Casein Digest Broth or Sabouraud Dextrose Broth) and the cells harvested by centrifugation, then washed and resuspended in appropriate sterile suspending fluid. The microbial suspensions used for inoculations should be adjusted to obtain a microbial count of about 1 ×

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1 Available from American Type Culture Collection, 10801 University Boulevard, Manassas, VA 20110-2209 (http://www.atcc.org).
10⁸ cfu/mL. Use the bacterial and yeast suspensions within 2 h, or within 24 h if stored between 2° and 8°. A stable spore suspension can be prepared and then may be maintained at 2°–8° for up to 7 days. [Note—The estimate of inoculum concentration may be obtained by turbidimetric procedures for the challenge microorganisms and later confirmed by plate count.]

GROWTH PROMOTION OF THE MEDIA

Media used in this procedure must be capable of supporting microbial growth. Test each batch of ready-prepared medium and each batch of medium prepared either from dehydrated medium or from the ingredients described.

For solid media, counts obtained must be at least 50% of the calculated value for a standardized inoculum. For a freshly prepared inoculum, growth of the microorganisms occurs comparable to that previously obtained with a previously tested and approved batch of medium.

Suitability of the Counting Method in the Presence of Product

Prepare a 10⁻ⁱ dilution by adding 1 mL of product (by volume) to 9 mL of saline or other neutralizing diluent. Continue this dilution scheme to 10⁻² and 10⁻³ dilution levels. Add an appropriate number of challenge organisms to each tube of diluted product, mix, and then plate a suitable volume from each dilution to yield less than 250 cfu/plate for bacteria and yeast (ideally between 25 and 250 cfu) or less than 80 cfu/plate for A. brasiliensis (ideally between 8 and 80 cfu). This plating should be performed minimally in duplicate (although a greater number of replicates can be useful to minimize variability in the plate count estimate). A positive control for this procedure is to introduce the same inocula into saline, and transfer similar volumes of saline to agar plates. A suitable recovery scheme is the one that provides at least 50% of this saline control count (averaged). If the diluted product exhibits antimicrobial properties, specific neutralizers may need to be incorporated into the diluents or the recovery media. See Validation of Microbial Recovery from Pharmacopeial Articles (1227) for more information.

The ability of the procedure to measure preservative efficacy may be compromised if the method suitability requires significant dilution (10⁻² or 10⁻³) as this will affect the measured recovery (e.g., it may be difficult to measure a 3 log unit reduction for a 10⁻¹–10⁰ inoculum). If no suitable neutralizing agent or method is found and method suitability requires significant dilution, a higher level of inoculum (e.g., 10⁻¹–10⁰) may be used so that a 3 log unit reduction can be measured. Reported recovery cannot be less than 1 cfu/plate on average (or 100 cfu/mL if 1 mL is plated in duplicate at the 10⁻² dilution). Membrane filtration may be used to filter larger volumes of dilutions to overcome this difficulty or to assist in the neutralization of antimicrobial properties.

Testing of Products

PRODUCT CATEGORIES

For the purpose of testing, compendial articles have been divided into four categories (see Table 1). The criteria of antimicrobial effectiveness for these products are a function of the route of administration. It is expected that formulations containing preservatives will meet minimal efficacy standards, whether packaged as multidoses or unit doses.

<table>
<thead>
<tr>
<th>Category</th>
<th>Product Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Injections; other parenterals including emulsions, otic products, sterile nasal products, and ophthalmic products made with aqueous bases or vehicles</td>
</tr>
<tr>
<td>2</td>
<td>Topically used products made with aqueous bases or vehicles; nonsterile nasal products and emulsions, including those applied to mucous membranes</td>
</tr>
<tr>
<td>3</td>
<td>Oral products other than antacids, made with aqueous bases or vehicles</td>
</tr>
<tr>
<td>4</td>
<td>Antacids made with an aqueous base</td>
</tr>
</tbody>
</table>

PROCEDURE

The procedure can be conducted either in five original containers if a sufficient volume of product is available in each container and if the product container can be entered aseptically (i.e., needle and syringe through an elastomeric rubber stopper), or in five sterile, capped bacteriological containers (inert relative to the antimicrobial agent(s)) of suitable size into which a sufficient volume of product has been transferred. Inoculate each container with one of the prepared and standardized inocula, and mix.

The volume of the suspension inoculum used is between 0.5% and 1.0% of the volume of the product to minimize potential effects on the product. The concentration of test microorganisms that is added to the product (Category 1, 2, or 3) is such that the final concentration of the test preparation after inoculation is between 1 × 10⁵ and 1 × 10⁷ cfu/mL of the product. For Category 4 products (antacids), the final concentration of the test preparation after inoculation is between 1 × 10³ and 1 × 10⁴ cfu/mL of the product.

The initial concentration of viable microorganisms in each test preparation is estimated based on the concentration of microorganisms in each of the standardized inocula as determined by the plate-count method. Incubate the inoculated containers at 22.5 ± 2.5°. Sample each container at the appropriate intervals (specified in Table 3). Record any changes observed in appearance at these intervals. Determine, by the plate-count procedure, the number of cfu present in each test preparation for the applicable intervals (see General Procedures in Microbial Enumeration Tests (61)). Plate counts will be conducted using a minimum of duplicate plates, with the cfu averaged before determination of deduced cfu/mL. If membrane filtration is used,
duplicate membrane filters will be used for each estimate. Using the calculated concentrations of cfu/mL present at the start of the test, calculate the change in \( \log_{10} \) values of the concentration of cfu/mL for each microorganism at the applicable test intervals, and express the changes in concentration in terms of log reductions. The log reduction is defined as the difference between the \( \log_{10} \) unit value of the starting concentration of cfu/mL in the suspension and the \( \log_{10} \) unit value of cfu/mL of the survivors at that time point.

### Table 2. Culture Conditions for Inoculum Preparation

<table>
<thead>
<tr>
<th>Organism</th>
<th>Suitable Medium</th>
<th>Incubation Temperature</th>
<th>Inoculum Incubation Time</th>
<th>Microbial Recovery Incubation Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Escherichia coli (ATCC No. 8739)</td>
<td>Soybean–Casein Digest Broth; Soybean–Casein Digest Agar</td>
<td>32.5 ± 2.5°C</td>
<td>18–24 h</td>
<td>3–5 days</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa (ATCC No. 9027)</td>
<td>Soybean–Casein Digest Broth; Soybean–Casein Digest Agar</td>
<td>32.5 ± 2.5°C</td>
<td>18–24 h</td>
<td>3–5 days</td>
</tr>
<tr>
<td>Staphylococcus aureus (ATCC No. 6538)</td>
<td>Soybean–Casein Digest Broth; Soybean–Casein Digest Agar</td>
<td>32.5 ± 2.5°C</td>
<td>18–24 h</td>
<td>3–5 days</td>
</tr>
<tr>
<td>Candida albicans (ATCC No. 10231)</td>
<td>Sabouraud Dextrose Agar; Sabouraud Dextrose Broth</td>
<td>22.5 ± 2.5°C</td>
<td>44–52 h</td>
<td>3–5 days</td>
</tr>
<tr>
<td>Aspergillus brasiliensis (ATCC No. 16404)</td>
<td>Sabouraud Dextrose Agar; Sabouraud Dextrose Broth</td>
<td>22.5 ± 2.5°C</td>
<td>6–10 days</td>
<td>3–7 days</td>
</tr>
</tbody>
</table>

### Criteria for Antimicrobial Effectiveness

The requirements for antimicrobial effectiveness are met if the criteria specified in Table 3 are met (see General Notices, 7. Test Results). "No increase" in counts is defined as NMT 0.5 \( \log_{10} \) unit more than the value to which it is compared.

### Table 3. Criteria for Tested Microorganisms

<table>
<thead>
<tr>
<th>For Category 1 Products</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteria</td>
</tr>
<tr>
<td>NLT 1.0 log reduction from the initial calculated count at 7 days, NLT 3.0 log reduction from the initial count at 14 days, and no increase from the 14 days’ count at 28 days</td>
</tr>
<tr>
<td>Yeast and molds</td>
</tr>
<tr>
<td>No increase from the initial calculated count at 7, 14, and 28 days</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>For Category 2 Products</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteria</td>
</tr>
<tr>
<td>NLT 2.0 log reduction from the initial count at 14 days, and no increase from the 14 days’ count at 28 days</td>
</tr>
<tr>
<td>Yeast and molds</td>
</tr>
<tr>
<td>No increase from the initial calculated count at 14 and 28 days</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>For Category 3 Products</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteria</td>
</tr>
<tr>
<td>NLT 1.0 log reduction from the initial count at 14 days, and no increase from the 14 days’ count at 28 days</td>
</tr>
<tr>
<td>Yeast and molds</td>
</tr>
<tr>
<td>No increase from the initial calculated count at 14 and 28 days</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>For Category 4 Products</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteria, yeast, and molds</td>
</tr>
<tr>
<td>No increase from the initial calculated count at 14 and 28 days</td>
</tr>
</tbody>
</table>

MICROBIOLOGICAL EXAMINATION OF NONSTERILE PRODUCTS: MICROBIAL ENUMERATION TESTS

### INTRODUCTION

The tests described hereafter will allow quantitative enumeration of mesophilic bacteria and fungi that may grow under aerobic conditions.

The tests are designed primarily to determine whether a substance or preparation complies with an established specification for microbiological quality. When used for such purposes, follow the instructions given below, including the number of samples to be taken, and interpret the results as stated below.

The methods are not applicable to products containing viable microorganisms as active ingredients.

Alternative microbiological procedures, including automated methods, may be used, provided that their equivalence to the Pharmacopeial method has been demonstrated.
GENERAL PROCEDURES

Carry out the determination under conditions designed to avoid extrinsic microbial contamination of the product to be examined. The precautions taken to avoid contamination must be such that they do not affect any microorganisms that are to be revealed in the test.

If the product to be examined has antimicrobial activity, this is, insofar as possible, removed or neutralized. If inactivators are used for this purpose, their efficacy and their absence of toxicity for microorganisms must be demonstrated.

If surface-active substances are used for sample preparation, their absence of toxicity for microorganisms and their compatibility with any inactivators used must be demonstrated.

ENUMERATION METHODS

Use the Membrane Filtration method or one of the Plate-Count Methods, as directed. The Most-Probable-Number (MPN) Method is generally the least accurate method for microbial counts; however, for certain product groups with very low bioburden, it may be the most appropriate method.

The choice of a method is based on factors such as the nature of the product and the required limit of microorganisms. The method chosen must allow testing of a sufficient sample size to judge compliance with the specification. The suitability of the chosen method must be established.

GROWTH PROMOTION TEST, SUITABILITY OF THE COUNTING METHOD AND NEGATIVE CONTROLS

General Considerations

The ability of the test to detect microorganisms in the presence of product to be tested must be established. Suitability must be confirmed if a change in testing performance or a change in the product that may affect the outcome of the test, is introduced.

Preparation of Test Strains

Use standardized stable suspensions of test strains or prepare as stated below. Seed-lot culture maintenance techniques (seed-lot systems) are used so that the viable microorganisms used for inoculation are not more than five passages removed from the original master seed-lot. Grow each of the bacterial and fungal test strains separately as described in Table 1.

Table 1. Preparation and Use of Test Microorganisms

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Preparation of Test Strain</th>
<th>Total Aerobic Microbial Count</th>
<th>Total Yeasts and Molds Count</th>
<th>Total Aerobic Microbial Count</th>
<th>Total Yeasts and Molds Count</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staphylococcus aureus</em> such as ATCC 6538, NCIMB 9518, CIP 4.83, or NBRC 13276</td>
<td>Soybean–Casein Digest Agar or Soybean–Casein Digest Broth 30°–35° 18–24 hours</td>
<td>Soybean–Casein Digest Agar and Soybean–Casein Digest Broth ≤100 cfu 30°–35° ≤3 days</td>
<td>Soybean–Casein Digest Agar or Soybean–Casein Digest Broth ≤100 cfu 30°–35° ≤3 days</td>
<td>Soybean–Casein Digest Agar or Soybean–Casein Digest Broth ≤100 cfu 30°–35° ≤3 days</td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> such as ATCC 9027, NCIMB 8626, CIP 82.118, or NBRC 13275</td>
<td>Soybean–Casein Digest Agar or Soybean–Casein Digest Broth 30°–35° 18–24 hours</td>
<td>Soybean–Casein Digest Agar and Soybean–Casein Digest Broth ≤100 cfu 30°–35° ≤3 days</td>
<td>Soybean–Casein Digest Agar or Soybean–Casein Digest Broth ≤100 cfu 30°–35° ≤3 days</td>
<td>Soybean–Casein Digest Agar or Soybean–Casein Digest Broth ≤100 cfu 30°–35° ≤3 days</td>
<td></td>
</tr>
<tr>
<td><em>Bacillus subtilis</em> such as ATCC 6633, NCIMB 8054, CIP 52.62, or NBRC 3134</td>
<td>Soybean–Casein Digest Agar or Soybean–Casein Digest Broth 30°–35° 18–24 hours</td>
<td>Soybean–Casein Digest Agar and Soybean–Casein Digest Broth ≤100 cfu 30°–35° ≤3 days</td>
<td>Soybean–Casein Digest Agar or Soybean–Casein Digest Broth ≤100 cfu 30°–35° ≤3 days</td>
<td>Soybean–Casein Digest Agar or Soybean–Casein Digest Broth ≤100 cfu 30°–35° ≤3 days</td>
<td></td>
</tr>
<tr>
<td><em>Candida albicans</em> such as ATCC 10231, NCPF 3179, IP 48.72, or NBRC 1594</td>
<td>Sabouraud Dextrose Agar or Sabouraud Dextrose Broth 20°–25° 2–3 days</td>
<td>Soybean–Casein Digest Agar ≤100 cfu 30°–35° ≤5 days</td>
<td>Sabouraud Dextrose Agar ≤100 cfu 20°–25° ≤5 days MPN: not applicable</td>
<td>Sabouraud Dextrose Agar ≤100 cfu 20°–25° ≤5 days MPN: not applicable</td>
<td></td>
</tr>
</tbody>
</table>
### Table 1. Preparation and Use of Test Microorganisms (continued)

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Preparation of Test Strain</th>
<th>Growth Promotion</th>
<th>Suitability of Counting Method in the Presence of Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Aspergillus brasiliensis</td>
<td>- Sabouraud Dextrose Agar</td>
<td>Soybean–Casein Di-gest Agar ≤100 cfu 30°–35° ≤5 days</td>
<td>Soybean–Casein Di-gest Agar ≤100 cfu 30°–35° ≤5 days</td>
</tr>
<tr>
<td>such as ATCC 16404, IMI 149007, IP 1431.83, or NBRC 9455</td>
<td>or Potato–Dextrose Agar 20°–25° 5–7 days, or until good sporulation is achieved</td>
<td>- Sabouraud Dextrose Agar ≤100 cfu 20°–25° ≤5 days</td>
<td>- Sabouraud Dextrose Agar ≤100 cfu 20°–25° ≤5 days</td>
</tr>
</tbody>
</table>

Use Buffered Sodium Chloride–Peptone Solution pH 7.0 or Phosphate Buffer Solution pH 7.2 to make test suspensions; to suspend A. brasiliensis spores, 0.05% of polysorbate 80 may be added to the buffer. Use the suspensions within 2 hours, or within 24 hours if stored between 2° and 8°. As an alternative to preparing and then diluting a fresh suspension of vegetative cells of A. brasiliensis or B. subtilis, a stable spore suspension is prepared and then an appropriate volume of the spore suspension is used for test inoculation. The stable spore suspension may be maintained at 2° to 8° for a validated period of time.

#### Negative Control

To verify testing conditions, a negative control is performed using the chosen diluent in place of the test preparation. There must be no growth of microorganisms. A negative control is also performed when testing the products as described under Testing of Products. A failed negative control requires an investigation.

#### Growth Promotion of the Media

Test each batch of ready-prepared medium and each batch of medium prepared either from dehydrated medium or from the ingredients described.

Inoculate portions/plates of Soybean–Casein Digest Broth and Soybean–Casein Digest Agar with a small number (not more than 100 cfu) of the microorganisms indicated in Table 1, using a separate portion/plate of medium for each. Inoculate plates of Sabouraud Dextrose Agar with a small number (not more than 100 cfu) of the microorganisms indicated in Table 1, using a separate plate of medium for each. Incubate according to the conditions described in Table 1.

For solid media, growth obtained must not differ by a factor greater than 2 from the calculated value for a standardized inoculum. For a freshly prepared inoculum, growth of the microorganisms comparable to that previously obtained with a previously tested and approved batch of medium occurs. Liquid media are suitable if clearly visible growth of the microorganisms comparable to that previously obtained with a previously tested and approved batch of medium occurs.

#### Suitability of the Counting Method in the Presence of Product

**PREPARATION OF THE SAMPLE**

The method for sample preparation depends on the physical characteristics of the product to be tested. If none of the procedures described below can be demonstrated to be satisfactory, a suitable alternative procedure must be developed.

**Water-Soluble Products**—Dissolve or dilute (usually a 1 in 10 dilution is prepared) the product to be examined in Buffered Sodium Chloride–Peptone Solution pH 7.0, Phosphate Buffer Solution pH 7.2, or Soybean–Casein Digest Broth. If necessary, adjust to a pH of 6 to 8. Further dilutions, where necessary, are prepared with the same diluent.

**Nonfatty Products Insoluble in Water**—Suspend the product to be examined (usually a 1 in 10 dilution is prepared) in Buffered Sodium Chloride–Peptone Solution pH 7.0, Phosphate Buffer Solution pH 7.2, or Soybean–Casein Digest Broth. A surface-active agent such as 1 g per L of polysorbate 80 may be added to assist the suspension of poorly wettable substances. If necessary, adjust to a pH of 6 to 8. Further dilutions, where necessary, are prepared with the same diluent.

**Fatty Products**—Dissolve in isopropyl myristate sterilized by filtration, or mix the product to be examined with the minimum necessary quantity of sterile polysorbate 80 or another noninhibitory sterile surface-active reagent heated, if necessary, to not more than 40° or, in exceptional cases, to not more than 45°. Mix carefully and if necessary maintain the temperature in a water bath. Add a sufficient quantity of the prewarmed chosen diluent to make a 1 in 10 dilution of the original product. Mix carefully, while maintaining the temperature for the shortest time necessary for the formation of an emulsion. Further serial 10-fold dilutions may be prepared using the chosen diluent containing a suitable concentration of sterile polysorbate 80 or another noninhibitory sterile surface-active reagent.

**Fluids or Solids in Aerosol Form**—Aseptically transfer the product into a membrane filter apparatus or a sterile container for further sampling. Use either the total contents or a defined number of metered doses from each of the containers tested.

**Transdermal Patches**—Remove the protective cover sheets (“release liners”) of the transdermal patches and place them, adhesive side upwards, on sterile glass or plastic trays. Cover the adhesive surface with a suitable sterile porous material (e.g., sterile gauze) to prevent the patches from sticking together, and transfer the patches to a suitable volume of the chosen diluent containing inactivators such as polysorbate 80 and/or lecithin. Shake the preparation vigorously for at least 30 minutes.

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*Not official text. Please refer to the currently official version of the applicable USP–NF or FCC standard for compliance purposes.*
INOCULATION AND DILUTION

Add to the sample prepared as directed above and to a control (with no test material included) a sufficient volume of the microbial suspension to obtain an inoculum of not more than 100 cfu. The volume of the suspension of the inoculum should not exceed 1% of the volume of diluted product.

To demonstrate acceptable microbial recovery from the product, the lowest possible dilution factor of the prepared sample must be used for the test. Where this is not possible due to antimicrobial activity or poor solubility, further appropriate protocols must be developed. If inhibition of growth by the sample cannot otherwise be avoided, the aliquot of the microbial suspension may be added after neutralization, dilution, or filtration.

NEUTRALIZATION/REMOVAL OF ANTIMICROBIAL ACTIVITY

The number of microorganisms recovered from the prepared sample diluted as described in Inoculation and Dilution and incubated following the procedure described in Recovery of Microorganisms in the Presence of Product, is compared to the number of microorganisms recovered from the control preparation.

If growth is inhibited (reduction by a factor greater than 2), then modify the procedure for the particular enumeration test to ensure the validity of the results. Modification of the procedure may include, for example,

1. An increase in the volume of the diluent or culture medium;
2. Incorporation of a specific or general neutralizing agents into the diluent;
3. Membrane filtration; or
4. A combination of the above measures.

Neutralizing Agents—Neutralizing agents may be used to neutralize the activity of antimicrobial agents (see Table 2). They may be added to the chosen diluent or the medium preferably before sterilization. If used, their efficacy and their absence of toxicity for microorganisms must be demonstrated by carrying out a blank with neutralizer and without product.

Table 2. Common Neutralizing Agents/Methods for Interfering Substances

<table>
<thead>
<tr>
<th>Interfering Substance</th>
<th>Potential Neutralizing Agents/Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutaraldehyde, mercurials</td>
<td>Sodium hydrogen sulfite (Sodium bisulfite)</td>
</tr>
<tr>
<td>Phenolics, alcohol, aldehydes, sorbate</td>
<td>Dilution</td>
</tr>
<tr>
<td>Aldehydes</td>
<td>Glycine</td>
</tr>
<tr>
<td>Quaternary ammonium compounds (QACs), parahydroxybenzoates (para-bens), bis-biguanides</td>
<td>Lecithin</td>
</tr>
<tr>
<td>QACs, iodine, parabens</td>
<td>Polysorbate</td>
</tr>
<tr>
<td>Mercurials</td>
<td>Thioglycollate</td>
</tr>
<tr>
<td>Mercurials, halogens, aldehydes</td>
<td>Thiosulfate</td>
</tr>
<tr>
<td>EDTA (edetate)</td>
<td>Mg or Ca ions</td>
</tr>
</tbody>
</table>

If no suitable neutralizing method can be found, it can be assumed that the failure to isolate the inoculated organism is attributable to the microbicidal activity of the product. This information serves to indicate that the article is not likely to be contaminated with the given species of the microorganism. However, it is possible that the product inhibits only some of the microorganisms specified herein, but does not inhibit others not included among the test strains or those for which the latter are not representative. Then, perform the test with the highest dilution factor compatible with microbial growth and the specific acceptance criterion.

RECOVERY OF MICROORGANISMS IN THE PRESENCE OF PRODUCT

For each of the microorganisms listed, separate tests are performed. Only microorganisms of the added test strain are counted.

Membrane Filtration—Use membrane filters having a nominal pore size not greater than 0.45 µm. The type of filter material is chosen in such a way that the bacteria-retaining efficiency is not affected by the components of the sample to be investigated. For each of the microorganisms listed, one membrane filter is used.

Transfer a suitable quantity of the sample prepared as described under Preparation of the Sample, Inoculation and Dilution, and Neutralization/Removal of Antimicrobial Activity (preferably representing 1 g of the product, or less if large numbers of cfu are expected) to the membrane filter, filter immediately, and rinse the membrane filter with an appropriate volume of diluent.

For the determination of total aerobic microbial count (TAMC), transfer the membrane filter to the surface of the Soybean–Casein Digest Agar. For the determination of total combined yeasts and molds count (TYMC), transfer the membrane to the surface of the Sabouraud Dextrose Agar: Incubate the plates as indicated in Table 1. Perform the counting.

Plate-Count Methods—Perform plate-count methods at least in duplicate for each medium, and use the mean count of the result.

Pour-Plate Method—For Petri dishes 9 cm in diameter, add to the dish 1 mL of the sample prepared as described under Preparation of the Sample, Inoculation and Dilution, and Neutralization/Removal of Antimicrobial Activity and 15 to 20 mL of Soybean–Casein Digest Agar or Sabouraud Dextrose Agar, both media maintained at not more than 45°. If larger Petri dishes are used, the amount of agar medium is increased accordingly. For each of the microorganisms listed in Table 1, at least two Petri dishes are used.
Incubate the plates as indicated in Table 1. Take the arithmetic mean of the counts per medium, and calculate the number of cfu in the original inoculum.

**Surface-Spread Method**—For Petri dishes 9 cm in diameter, add 15 to 20 mL of Soybean–Casein Digest Agar or Sabouraud Dextrose Agar at about 45° to each Petri dish, and allow to solidify. If larger Petri dishes are used, the volume of the agar is increased accordingly. Dry the plates, for example, in a laminar-airflow cabinet or in an incubator. For each of the microorganisms listed in Table 1, at least two Petri dishes are used. Spread a measured volume of not less than 0.1 mL of the sample, prepared as directed under Preparation of the Sample, Inoculation and Dilution, and Neutralization/Removal of Antimicrobial Activity over the surface of the medium. Incubate and count as directed for Pour-Plate Method.

**Most-Probable-Number (MPN) Method**—The precision and accuracy of the MPN Method is less than that of the Membrane Filtration method or the Plate-Count Method. Unreliable results are obtained particularly for the enumeration of molds. For these reasons, the MPN Method is reserved for the enumeration of TAMC in situations where no other method is available. If the use of the method is justified, proceed as follows.

Prepare a series of at least three serial 10-fold dilutions of the product as described for Preparation of the Sample, Inoculation and Dilution, and Neutralization/Removal of Antimicrobial Activity. From each level of dilution, three aliquots of 1 g or 1 mL are used to inoculate three tubes with 9 to 10 mL of Soybean–Casein Digest Broth. If necessary a surface-active agent such as polysorbate 80, or an inactivator of antimicrobial agents may be added to the medium. Thus, if three levels of dilution are prepared, nine tubes are inoculated.

Incubate all tubes at 30° to 35° for not more than 3 days. If reading of the results is difficult or uncertain owing to the nature of the product to be examined, subculture in the same broth or in Soybean–Casein Digest Agar for 1 to 2 days at the same temperature, and use these results. From Table 3, determine the most probable number of microorganisms per g or mL of the product to be examined.

<table>
<thead>
<tr>
<th>Table 3. Most-Probable-Number Values of Microorganisms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Observed Combinations of Numbers of Tubes Showing Growth in Each Set</td>
</tr>
<tr>
<td>Number of g or mL of Product per Tube</td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td>0</td>
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<tr>
<td>0</td>
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</tbody>
</table>
### Table 3. Most-Probable-Number Values of Microorganisms (continued)

<table>
<thead>
<tr>
<th>Observed Combinations of Numbers of Tubes Showing Growth in Each Set</th>
<th>MPN per g or per mL of Product</th>
<th>95% Confidence Limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of g or mL of Product per Tube</td>
<td>64</td>
<td>16–181</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>2</td>
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</tbody>
</table>

### RESULTS AND INTERPRETATION

When verifying the suitability of the Membrane Filtration method or the Plate-Count Method, a mean count of any of the test organisms not differing by a factor greater than 2 from the value of the control defined in Inoculation and Dilution in the absence of product must be obtained. When verifying the suitability of the MPN Method, the calculated value from the inoculum must be within 95% confidence limits of the results obtained with the control.

If the above criteria cannot be met for one of more of the organisms tested with any of the described methods, the method and test conditions that come closest to the criteria are used to test the product.

### TESTING OF PRODUCTS

#### Amount Used for the Test

Unless otherwise directed, use 10 g or 10 mL of the product to be examined taken with the precautions referred to above. For fluids or solids in aerosol form, sample 10 containers. For transdermal patches, sample 10 patches.

The amount to be tested may be reduced for active substances that will be formulated in the following conditions: the amount per dosage unit (e.g., tablet, capsule, injection) is less than or equal to 1 mg, or the amount per g or mL (for preparations not presented in dose units) is less than 1 mg. In these cases, the amount of sample to be tested is not less than the amount present in 10 dosage units or 10 g or 10 mL of the product.

For materials used as active substances where the sample quantity is limited or batch size is extremely small (i.e., less than 1000 mL or 1000 g), the amount tested shall be 1% of the batch unless a lesser amount is prescribed or justified and authorized. For products where the total number of entities in a batch is less than 200 (e.g., samples used in clinical trials), the sample size may be reduced to two units, or one unit if the size is less than 100.

Select the sample(s) at random from the bulk material or from the available containers of the preparation. To obtain the required quantity, mix the contents of a sufficient number of containers to provide the sample.

#### Examination of the Product

**MEMBRANE FILTRATION**

Use a filtration apparatus designed to allow the transfer of the filter to the medium. Prepare the sample using a method that has been shown to be suitable as described in Growth Promotion Test and Suitability of the Counting Method, transfer the appropriate amount to each of two membrane filters, and filter immediately. Wash each filter following the procedure shown to be suitable.

For the determination of TAMC, transfer one of the membrane filters to the surface of Soybean–Casein Digest Agar. For the determination of TYMC, transfer the other membrane to the surface of Sabouraud Dextrose Agar. Incubate the plate of Soybean–Casein Digest Agar at 30° to 35° for 3 to 5 days and the plate of Sabouraud Dextrose Agar at 20° to 25° for 5 to 7 days. Calculate the number of cfu per g or per mL of product.

When examining transdermal patches, separately filter 10% of the volume of the preparation described for Preparation of the Sample through each of two sterile filter membranes. Transfer one membrane to Soybean–Casein Digest Agar for TAMC and the other membrane to Sabouraud Dextrose Agar for TYMC.
PLATE-COUNT METHODS

**Pour-Plate Method**—Prepare the sample using a method that has been shown to be suitable as described in *Growth Promotion Test and Suitability of the Counting Method*. Prepare for each medium at least two Petri dishes for each level of dilution. Incubate the plates of *Soybean–Casein Digest Agar* at 30° to 35° for 3 to 5 days and the plates of *Sabouraud Dextrose Agar* at 20° to 25° for 5 to 7 days. Select the plates corresponding to a given dilution and showing the highest number of colonies less than 250 for TAMC and 50 for TYMC. Take the arithmetic mean per culture medium of the counts, and calculate the number of cfu per g or per mL of product.

**Surface-Spread Method**—Prepare the sample using a method that has been shown to be suitable as described in *Growth Promotion Test and Suitability of the Counting Method*. Prepare at least two Petri dishes for each medium and each level of dilution. For incubation and calculation of the number of cfu, proceed as directed for the *Pour-Plate Method*.

**MOST-PROBABLE-NUMBER METHOD**

Prepare and dilute the sample using a method that has been shown to be suitable as described in *Growth Promotion Test and Suitability of the Counting Method*. Incubate all tubes for 3 to 5 days at 30° to 35°. Subculture if necessary, using the procedure shown to be suitable. Record for each level of dilution the number of tubes showing microbial growth. Determine the most probable number of microorganisms per g or mL of the product to be examined from Table 3.

**Interpretation of the Results**

The total aerobic microbial count (TAMC) is considered to be equal to the number of cfu found using *Soybean–Casein Digest Agar*; if colonies of fungi are detected on this medium, they are counted as part of TAMC. The total combined yeasts and molds count (TYMC) is considered to be equal to the number of cfu found using *Sabouraud Dextrose Agar*; if colonies of bacteria are detected on this medium, they are counted as part of TYMC. When the TYMC is expected to exceed the acceptance criterion due to the bacterial growth, *Sabouraud Dextrose Agar* containing antibiotics may be used. If the count is carried out by the *MPN Method*, the calculated value is TAMC.

When an acceptance criterion for microbiological quality is prescribed, it is interpreted as follows:

- $10^1$ cfu: maximum acceptable count = 20;
- $10^2$ cfu: maximum acceptable count = 200;
- $10^3$ cfu: maximum acceptable count = 2000;

and so forth.

The recommended solutions and media are described in *Tests for Specified Microorganisms* (62).

### 62 MICROBIOLOGICAL EXAMINATION OF NONSTERILE PRODUCTS: TESTS FOR SPECIFIED MICROORGANISMS

**INTRODUCTION**

The tests described hereafter will allow determination of the absence of, or limited occurrence of, specified microorganisms that may be detected under the conditions described. The tests are designed primarily to determine whether a substance or preparation complies with an established specification for microbiological quality. When used for such purposes, follow the instructions given below, including the number of samples to be taken, and interpret the results as stated below.

Alternative microbiological procedures, including automated methods, may be used, provided that their equivalence to the Pharmacopeial method has been demonstrated.

**GENERAL PROCEDURES**

The preparation of samples is carried out as described in *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* (61).

If the product to be examined has antimicrobial activity, this is insofar as possible removed or neutralized as described in *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* (61).

If surface-active substances are used for sample preparation, their absence of toxicity for microorganisms and their compatibility with any inactivators used must be demonstrated as described in *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* (61).
GROWTH-PROMOTING AND INHIBITORY PROPERTIES OF THE MEDIA, SUITABILITY OF THE TEST AND NEGATIVE CONTROLS

The ability of the test to detect microorganisms in the presence of the product to be tested must be established. Suitability must be confirmed if a change in testing performance or a change in the product that may affect the outcome of the test is introduced.

Preparation of Test Strains

Use standardized stable suspensions of test strains as stated below. Seed-lot culture maintenance techniques (seed-lot systems) are used so that the viable microorganisms used for inoculation are not more than five passages removed from the original master seed-lot.

AEROBIC MICROORGANISMS

Grow each of the bacterial test strains separately in containers containing Soybean–Casein Digest Broth or on Soybean–Casein Digest Agar at 30° to 35° for 18 to 24 hours. Grow the test strain for Candida albicans separately on Sabouraud Dextrose Agar or in Sabouraud Dextrose Broth at 20° to 25° for 2 to 3 days.

<table>
<thead>
<tr>
<th>Test Strains</th>
<th>Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staphylococcus aureus</td>
<td>such as ATCC 6538, NCIMB 9518, CIP 4.83, or NBRC 13276</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>such as ATCC 9027, NCIMB 8626, CIP 82.118, or NBRC 13275</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>such as ATCC 8739, NCIMB 8545, CIP 53.126, or NBRC 3972</td>
</tr>
<tr>
<td>Salmonella enterica subsp. enterica serovar Typhimurium or, as an alternative, Salmonella enterica subsp. enterica serovar Abony</td>
<td>such as NBRC 100797, NCTC 6017, or CIP 80.39</td>
</tr>
<tr>
<td>Candida albicans</td>
<td>such as ATCC 10231, NCFP 3179, IP 48.72, or NBRC 1594</td>
</tr>
</tbody>
</table>

Use Buffered Sodium Chloride–Peptone Solution pH 7.0 or Phosphate Buffer Solution pH 7.2 to make test suspensions. Use the suspensions within 2 hours or within 24 hours if stored at 2° to 8°.

CLOSTRIDIA

Use Clostridium sporogenes such as ATCC 11437 (NBRC 14293, NCIMB 12343, CIP 100651) or ATCC 19404 (NCTC 532 or CIP 79.3). Grow the clostridial test strain under anaerobic conditions in Reinforced Medium for Clostridia at 30° to 35° for 24 to 48 hours. As an alternative to preparing and then diluting down a fresh suspension of vegetative cells of Cl. sporogenes, a stable spore suspension is used for test inoculation. The stable spore suspension may be maintained at 2° to 8° for a validated period.

Negative Control

To verify testing conditions, a negative control is performed using the chosen diluent in place of the test preparation. There must be no growth of microorganisms. A negative control is also performed when testing the products as described under Testing of Products. A failed negative control requires an investigation.

Growth Promotion and Inhibitory Properties of the Media

Test each batch of ready-prepared medium and each batch of medium prepared either from dehydrated medium or from ingredients. Verify suitable properties of relevant media as described in Table 1.

<table>
<thead>
<tr>
<th>Test Medium</th>
<th>Property</th>
<th>Test Strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test for bile-tolerant Gram-negative bacteria</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enterobacteria Enrichment Broth Mossel</td>
<td>Growth promoting</td>
<td>E. coli</td>
</tr>
<tr>
<td></td>
<td>Inhibitory</td>
<td>S. aureus</td>
</tr>
<tr>
<td>Violet Red Bile Glucose Agar</td>
<td>Growth promoting + Indicative</td>
<td>E. coli</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P. aeruginosa</td>
</tr>
<tr>
<td>Test for Escherichia coli</td>
<td>Growth promoting</td>
<td>E. coli</td>
</tr>
<tr>
<td></td>
<td>Inhibitory</td>
<td>S. aureus</td>
</tr>
<tr>
<td>MacConkey Broth</td>
<td>Growth promoting + Indicative</td>
<td>E. coli</td>
</tr>
</tbody>
</table>
Table 1. Growth Promoting, Inhibitory, and Indicative Properties of Media (continued)

<table>
<thead>
<tr>
<th>Test/Medium</th>
<th>Property</th>
<th>Test Strains</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Rappaport Vassiliadis Salmonella Enrichment Broth</em></td>
<td>Growth promoting</td>
<td><em>Salmonella enterica subsp. enterica serovar Typhimurium</em> or</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Inhibitory</td>
<td><em>S. aureus</em></td>
</tr>
<tr>
<td><em>Xylose Lysine Deoxycholate Agar</em></td>
<td>Growth promoting + Indicative</td>
<td><em>Salmonella enterica subsp. enterica serovar Typhimurium</em> or</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Salmonella enterica subsp. enterica serovar Abony</em></td>
</tr>
<tr>
<td><em>Cetrimide Agar</em></td>
<td>Growth promoting</td>
<td><em>P. aeruginosa</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Mannitol Salt Agar</em></td>
<td>Growth promoting + Indicative</td>
<td><em>S. aureus</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Columbia Agar</em></td>
<td>Inhibitory</td>
<td><em>E. coli</em></td>
</tr>
<tr>
<td><em>Reinforced Medium for Clostridia</em></td>
<td>Growth promoting</td>
<td><em>Cl. sporogenes</em></td>
</tr>
<tr>
<td><em>Columbia Agar</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Columbia Agar</em></td>
<td>Growth promoting</td>
<td><em>Cl. sporogenes</em></td>
</tr>
<tr>
<td><em>Sabouraud Dextrose Broth</em></td>
<td>Growth promoting</td>
<td><em>C. albicans</em></td>
</tr>
<tr>
<td><em>Sabouraud Dextrose Agar</em></td>
<td>Growth promoting + Indicative</td>
<td><em>C. albicans</em></td>
</tr>
</tbody>
</table>

**TEST FOR GROWTH-PROMOTING PROPERTIES, LIQUID MEDIA**

Inoculate a portion of the appropriate medium with a small number (not more than 100 cfu) of the appropriate microorganism. Incubate at the specified temperature for not more than the shortest period of time specified in the test. Clearly visible growth of the microorganism comparable to that previously obtained with a previously tested and approved batch of medium occurs.

**TEST FOR GROWTH-PROMOTING PROPERTIES, SOLID MEDIA**

Perform Surface-Spread Method (see Plate-Count Methods under Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests (61)), inoculating each plate with a small number (not more than 100 cfu) of the appropriate microorganism. Incubate at the specified temperature for not more than the shortest period of time specified in the test. Growth of the microorganism comparable to that previously obtained with a previously tested and approved batch of medium occurs.

**TEST FOR INHIBITORY PROPERTIES, LIQUID OR SOLID MEDIA**

Inoculate the appropriate medium with at least 100 cfu of the appropriate microorganism. Incubate at the specified temperature for not less than the longest period of time specified in the test. No growth of the test microorganism occurs.

**TEST FOR INDICATIVE PROPERTIES**

Perform Surface-Spread Method (see Plate-Count Methods under Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests (61)), inoculating each plate with a small number (not more than 100 cfu) of the appropriate microorganism. Incubate at the specified temperature for a period of time within the range specified in the test. Colonies are comparable in appearance and indication reactions to those previously obtained with a previously tested and approved batch of medium.

**Suitability of the Test Method**

For each new product to be tested perform sample preparation as described in the relevant paragraph under Testing of Products. At the time of mixing, add each test strain in the prescribed growth medium. Inoculate the test strains individually. Use a number of microorganisms equivalent to not more than 100 cfu in the inoculated test preparation.

Perform the test as described in the relevant paragraph under Testing of Products using the shortest incubation period prescribed.

The specified microorganisms must be detected with the indication reactions as described under Testing of Products. Any antimicrobial activity of the product necessitates a modification of the test procedure (see Neutralization/Removal of Antimicrobial Activity under Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests (61)).
For a given product, if the antimicrobial activity with respect to a microorganism for which testing is prescribed cannot be neutralized, then it is to be assumed that the inhibited microorganism will not be present in the product.

**TESTING OF PRODUCTS**

**Bile-Tolerant Gram-Negative Bacteria**

**SAMPLE PREPARATION AND PRE-INCUBATION**

Prepare a sample using a 1 in 10 dilution of not less than 1 g of the product to be examined as described in Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests (61), but using Soybean–Casein Digest Broth as the chosen diluent, mix, and incubate at 20° to 25° for a time sufficient to resuscitate the bacteria but not sufficient to encourage multiplication of the organisms (usually 2 hours but not more than 5 hours).

**TEST FOR ABSENCE**

Unless otherwise prescribed, use the volume corresponding to 1 g of the product, as prepared in **Sample Preparation and Pre-Incubation**, to inoculate Enterobacteria Enrichment Broth Mossel. Incubate at 30° to 35° for 24 to 48 hours. Subculture on plates of Violet Red Bile Glucose Agar. Incubate at 30° to 35° for 18 to 24 hours.

The product complies with the test if there is no growth of colonies.

**QUANTITATIVE TEST**

**Selection and Subculture**—Inoculate suitable quantities of Enterobacteria Enrichment Broth Mossel with the preparation as directed under **Sample Preparation and Pre-Incubation** and/or dilutions of it containing respectively 0.1 g, 0.01 g, and 0.001 g (or 0.1 mL, 0.01 mL, and 0.001 mL) of the product to be examined. Incubate at 30° to 35° for 24 to 48 hours. Subculture each of the cultures on a plate of Violet Red Bile Glucose Agar. Incubate at 30° to 35° for 18 to 24 hours.

**Interpretation**—Growth of colonies constitutes a positive result. Note the smallest quantity of the product that gives a positive result and the largest quantity that gives a negative result. Determine from Table 2 the probable number of bacteria.

<table>
<thead>
<tr>
<th>Results for Each Quantity of Product</th>
<th>Probable Number of Bacteria per g or mL of Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 g or 0.1 mL</td>
<td>0.01 g or 0.01 mL</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Escherichia coli**

**SAMPLE PREPARATION AND PRE-INCUBATION**

Prepare a sample using a 1 in 10 dilution of not less than 1 g of the product to be examined as described in Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests (61), and use 10 mL or the quantity corresponding to 1 g or 1 mL, to inoculate a suitable amount (determined as described under Suitability of the Test Method) of Soybean–Casein Digest Broth, mix, and incubate at 30° to 35° for 18 to 24 hours.

**SELECTION AND SUBCULTURE**

Shake the container, transfer 1 mL of Soybean–Casein Digest Broth to 100 mL of MacConkey Broth, and incubate at 42° to 44° for 24 to 48 hours. Subculture on a plate of MacConkey Agar at 30° to 35° for 18 to 72 hours.

**INTERPRETATION**

Growth of colonies indicates the possible presence of E. coli. This is confirmed by identification tests. The product complies with the test if no colonies are present or if the identification tests are negative.

**Salmonella**

**SAMPLE PREPARATION AND PRE-INCUBATION**

Prepare the product to be examined as described in Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests (61), and use the quantity corresponding to not less than 10 g or 10 mL to inoculate a suitable amount (determined as
described under *Suitability of the Test Method* of *Soybean–Casein Digest Broth*, mix, and incubate at 30° to 35° for 18 to 24 hours.

**SELECTION AND SUBCULTURE**

Transfer 0.1 mL of *Soybean–Casein Digest Broth* to 10 mL of *Rappaport Vassiliadis Salmonella Enrichment Broth*, and incubate at 30° to 35° for 18 to 24 hours. Subculture on plates of *Xylose Lysine Deoxycholate Agar*. Incubate at 30° to 35° for 18 to 48 hours.

**INTERPRETATION**

The possible presence of *Salmonella* is indicated by the growth of well-developed, red colonies, with or without black centers. This is confirmed by identification tests. The product complies with the test if colonies of the types described are not present or if the confirmatory identification tests are negative.

*Pseudomonas aeruginosa*

**SAMPLE PREPARATION AND PRE-INCUBATION**

Prepare a sample using a 1 in 10 dilution of not less than 1 g of the product to be examined as described in *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* (61), and use 10 mL or the quantity corresponding to 1 g or 1 mL to inoculate a suitable amount (determined as described under *Suitability of the Test Method*) of *Soybean–Casein Digest Broth*, and mix. When testing transdermal patches, filter the volume of sample corresponding to one patch of the preparation (see *Transdermal Patches* under *Preparation of the Sample* in *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* (61)) through a sterile filter membrane, and place in 100 mL of *Soybean–Casein Digest Broth*. Incubate at 30° to 35° for 18 to 24 hours.

**SELECTION AND SUBCULTURE**

Subculture on a plate of *Cetrimide Agar*, and incubate at 30° to 35° for 18 to 72 hours.

**INTERPRETATION**

Growth of colonies indicates the possible presence of *P. aeruginosa*. This is confirmed by identification tests. The product complies with the test if colonies are not present or if the confirmatory identification tests are negative.

*Staphylococcus aureus*

**SAMPLE PREPARATION AND PRE-INCUBATION**

Prepare a sample using a 1 in 10 dilution of not less than 1 g of the product to be examined as described in *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* (61), and use 10 mL or the quantity corresponding to 1 g or 1 mL to inoculate a suitable amount (determined as described under *Suitability of the Test Method*) of *Soybean–Casein Digest Broth*, and homogenize. When testing transdermal patches, filter the volume of sample corresponding to one patch of the preparation (see *Transdermal Patches* under *Preparation of the Sample* in *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* (61)) through a sterile filter membrane, and place in 100 mL of *Soybean–Casein Digest Broth*. Incubate at 30° to 35° for 18 to 24 hours.

**SELECTION AND SUBCULTURE**

Subculture on a plate of *Mannitol Salt Agar*, and incubate at 30° to 35° for 18 to 72 hours.

**INTERPRETATION**

The possible presence of *S. aureus* is indicated by the growth of yellow or white colonies surrounded by a yellow zone. This is confirmed by identification tests. The product complies with the test if colonies of the types described are not present or if the confirmatory identification tests are negative.

*Clostridia*

**SAMPLE PREPARATION AND HEAT TREATMENT**

Prepare a sample using a 1 in 10 dilution (with a minimum total volume of 20 mL) of not less than 2 g or 2 mL of the product to be examined as described in *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* (61). Divide the sample into two portions of at least 10 mL. Heat one portion at 80° for 10 minutes, and cool rapidly. Do not heat the other portion.
**Selection and Subculture**

Use 10 mL or the quantity corresponding to 1 g or 1 mL of the product to be examined of both portions to inoculate suitable amounts (determined as described under Suitability of the Test Method) of Reinforced Medium for Clostridia. Incubate under anaerobic conditions at 30° to 35° for 48 hours. After incubation, make subcultures from each container on Columbia Agar, and incubate under anaerobic conditions at 30° to 35° for 48 to 72 hours.

**Interpretation**

The occurrence of anaerobic growth of rods (with or without endospores) giving a negative catalase reaction indicates the presence of Clostridia. This is confirmed by identification tests. The product complies with the test if colonies of the types described are not present or if the confirmatory identification tests are negative.

*Candida albicans*

**Sample Preparation and Pre-Incubation**

Prepare the product to be examined as described in Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests (61), and use 10 mL or the quantity corresponding to not less than 1 g or 1 mL, to inoculate 100 mL of Sabouraud Dextrose Broth, and mix. Incubate at 30° to 35° for 3 to 5 days.

**Selection and Subculture**

Subculture on a plate of Sabouraud Dextrose Agar, and incubate at 30° to 35° for 24 to 48 hours.

**Interpretation**

Growth of white colonies may indicate the presence of *C. albicans*. This is confirmed by identification tests. The product complies with the test if such colonies are not present or if the confirmatory identification tests are negative.

**Recommended Solutions and Culture Media**

[NOTE—This section is given for information.]

The following solutions and culture media have been found satisfactory for the purposes for which they are prescribed in the test for microbial contamination in the Pharmacopeia. Other media may be used provided that their suitability can be demonstrated.

**Stock Buffer Solution**—Transfer 34 g of potassium dihydrogen phosphate to a 1000-mL volumetric flask, dissolve in 500 mL of Purified Water, adjust with sodium hydroxide to a pH of 7.2 ± 0.2, add Purified Water to volume, and mix. Dispense in containers, and sterilize. Store at a temperature of 2° to 8°.

**Phosphate Buffer Solution pH 7.2**—Prepare a mixture of Purified Water and Stock Buffer Solution (800:1 v/v), and sterilize.

**Buffered Sodium Chloride–Peptone Solution pH 7.0**

<table>
<thead>
<tr>
<th>Buffered Sodium Chloride–Peptone Solution pH 7.0</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium Dihydrogen Phosphate</td>
<td>3.6 g</td>
</tr>
<tr>
<td>Disodium Hydrogen Phosphate Dihydrate</td>
<td>7.2 g (equivalent to 0.067 M phosphate)</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>4.3 g</td>
</tr>
<tr>
<td>Peptone (meat or casein)</td>
<td>1.0 g</td>
</tr>
<tr>
<td>Purified Water</td>
<td>1000 mL</td>
</tr>
</tbody>
</table>

Sterilize in an autoclave using a validated cycle.

**Soybean–Casein Digest Broth**

<table>
<thead>
<tr>
<th>Soybean–Casein Digest Broth</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Pancreatic Digest of Casein</td>
<td>17.0 g</td>
</tr>
<tr>
<td>Papic Digest of Soybean</td>
<td>3.0 g</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Dibasic Hydrogen Phosphate</td>
<td>2.5 g</td>
</tr>
<tr>
<td>Glucose Monohydrate</td>
<td>2.5 g</td>
</tr>
<tr>
<td>Purified Water</td>
<td>1000 mL</td>
</tr>
</tbody>
</table>

Adjust the pH so that after sterilization it is 7.3 ± 0.2 at 25°. Sterilize in an autoclave using a validated cycle.

Published on March 26, 2020
### Soybean–Casein Digest Agar

<table>
<thead>
<tr>
<th>Component</th>
<th>Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pancreatic Digest of Casein</td>
<td>15.0 g</td>
</tr>
<tr>
<td>Papaic Digest of Soybean</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0 g</td>
</tr>
<tr>
<td>Purified Water</td>
<td>1000 mL</td>
</tr>
</tbody>
</table>

Adjust the pH so that after sterilization it is 7.3 ± 0.2 at 25°. Sterilize in an autoclave using a validated cycle.

### Sabouraud Dextrose Agar

<table>
<thead>
<tr>
<th>Component</th>
<th>Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dextrose</td>
<td>40.0 g</td>
</tr>
<tr>
<td>Mixture of Peptic Digest of Animal Tissue and Pancreatic Digest of Casein (1:1)</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0 g</td>
</tr>
<tr>
<td>Purified Water</td>
<td>1000 mL</td>
</tr>
</tbody>
</table>

Adjust the pH so that after sterilization it is 5.6 ± 0.2 at 25°. Sterilize in an autoclave using a validated cycle.

### Potato Dextrose Agar

<table>
<thead>
<tr>
<th>Component</th>
<th>Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infusion from potatoes</td>
<td>200 g</td>
</tr>
<tr>
<td>Dextrose</td>
<td>20.0 g</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0 g</td>
</tr>
<tr>
<td>Purified Water</td>
<td>1000 mL</td>
</tr>
</tbody>
</table>

Adjust the pH so that after sterilization it is 5.6 ± 0.2 at 25°. Sterilize in an autoclave using a validated cycle.

### Sabouraud Dextrose Broth

<table>
<thead>
<tr>
<th>Component</th>
<th>Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dextrose</td>
<td>20.0 g</td>
</tr>
<tr>
<td>Mixture of Peptic Digest of Animal Tissue and Pancreatic Digest of Casein (1:1)</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Purified Water</td>
<td>1000 mL</td>
</tr>
</tbody>
</table>

Adjust the pH so that after sterilization it is 5.6 ± 0.2 at 25°. Sterilize in an autoclave using a validated cycle.

### Enterobacteria Enrichment Broth Mossel

<table>
<thead>
<tr>
<th>Component</th>
<th>Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pancreatic Digest of Gelatin</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Glucose Monohydrate</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Dehydrated Ox Bile</td>
<td>20.0 g</td>
</tr>
<tr>
<td>Potassium Dihydrogen Phosphate</td>
<td>2.0 g</td>
</tr>
<tr>
<td>Disodium Hydrogen Phosphate Dihydrate</td>
<td>8.0 g</td>
</tr>
<tr>
<td>Brilliant Green</td>
<td>15 mg</td>
</tr>
<tr>
<td>Purified Water</td>
<td>1000 mL</td>
</tr>
</tbody>
</table>

Adjust the pH so that after heating it is 7.2 ± 0.2 at 25°. Heat at 100° for 30 minutes, and cool immediately.

### Violet Red Bile Glucose Agar

<table>
<thead>
<tr>
<th>Component</th>
<th>Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast Extract</td>
<td>3.0 g</td>
</tr>
<tr>
<td>Pancreatic Digest of Gelatin</td>
<td>7.0 g</td>
</tr>
<tr>
<td>Bile Salts</td>
<td>1.5 g</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Glucose Monohydrate</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0 g</td>
</tr>
<tr>
<td>Neutral Red</td>
<td>30 mg</td>
</tr>
</tbody>
</table>
**Violet Red Bile Glucose Agar**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crystal Violet</td>
<td>2 mg</td>
</tr>
<tr>
<td>Purified Water</td>
<td>1000 mL</td>
</tr>
</tbody>
</table>

Adjust the pH so that after heating it is 7.4 ± 0.2 at 25°. Heat to boiling; do not heat in an autoclave.

**MacConkey Broth**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pancreatic Digest of Gelatin</td>
<td>20.0 g</td>
</tr>
<tr>
<td>Lactose Monohydrate</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Dehydrated Ox Bile</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Bromocresol Purple</td>
<td>10 mg</td>
</tr>
<tr>
<td>Purified Water</td>
<td>1000 mL</td>
</tr>
</tbody>
</table>

Adjust the pH so that after sterilization it is 7.3 ± 0.2 at 25°. Sterilize in an autoclave using a validated cycle.

**MacConkey Agar**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pancreatic Digest of Gelatin</td>
<td>17.0 g</td>
</tr>
<tr>
<td>Peptones (meat and casein)</td>
<td>3.0 g</td>
</tr>
<tr>
<td>Lactose Monohydrate</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Bile Salts</td>
<td>1.5 g</td>
</tr>
<tr>
<td>Agar</td>
<td>13.5 g</td>
</tr>
<tr>
<td>Neutral Red</td>
<td>30.0 mg</td>
</tr>
<tr>
<td>Crystal Violet</td>
<td>1 mg</td>
</tr>
<tr>
<td>Purified Water</td>
<td>1000 mL</td>
</tr>
</tbody>
</table>

Adjust the pH so that after sterilization it is 7.1 ± 0.2 at 25°. Boil for 1 minute with constant shaking, then sterilize in an autoclave using a validated cycle.

**Rappaport Vassiliadis Salmonella Enrichment Broth**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soya Peptone</td>
<td>4.5 g</td>
</tr>
<tr>
<td>Magnesium Chloride Hexahydrate</td>
<td>29.0 g</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>8.0 g</td>
</tr>
<tr>
<td>Dipotassium Phosphate</td>
<td>0.4 g</td>
</tr>
<tr>
<td>Potassium Dihydrogen Phosphate</td>
<td>0.6 g</td>
</tr>
<tr>
<td>Malachite Green</td>
<td>0.036 g</td>
</tr>
<tr>
<td>Purified Water</td>
<td>1000 mL</td>
</tr>
</tbody>
</table>

Dissolve, warming slightly. Sterilize in an autoclave using a validated cycle, at a temperature not exceeding 115°. The pH is to be 5.2 ± 0.2 at 25° after heating and autoclaving.

**Xylose Lysine Deoxycholate Agar**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xylose</td>
<td>3.5 g</td>
</tr>
<tr>
<td>L-Lysine</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Lactose Monohydrate</td>
<td>7.5 g</td>
</tr>
<tr>
<td>Sucrose</td>
<td>7.5 g</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>3.0 g</td>
</tr>
<tr>
<td>Phenol Red</td>
<td>80 mg</td>
</tr>
<tr>
<td>Agar</td>
<td>13.5 g</td>
</tr>
<tr>
<td>Sodium Deoxycholate</td>
<td>2.5 g</td>
</tr>
</tbody>
</table>
Xylose Lysine Deoxycholate Agar

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium Thiosulfate</td>
<td>6.8 g</td>
</tr>
<tr>
<td>Ferric Ammonium Citrate</td>
<td>0.8 g</td>
</tr>
<tr>
<td>Purified Water</td>
<td>1000 mL</td>
</tr>
</tbody>
</table>

Adjust the pH so that after heating it is 7.4 ± 0.2 at 25°. Heat to boiling, cool to 50°, and pour into Petri dishes. Do not heat in an autoclave.

Cetrimide Agar

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pancreatic Digest of Gelatin</td>
<td>20.0 g</td>
</tr>
<tr>
<td>Magnesium Chloride</td>
<td>1.4 g</td>
</tr>
<tr>
<td>Dipotassium Sulfate</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Cetrimide</td>
<td>0.3 g</td>
</tr>
<tr>
<td>Agar</td>
<td>13.6 g</td>
</tr>
<tr>
<td>Purified Water</td>
<td>1000 mL</td>
</tr>
<tr>
<td>Glycerol</td>
<td>10.0 mL</td>
</tr>
</tbody>
</table>

Heat to boiling for 1 minute with shaking. Adjust the pH so that after sterilization it is 7.2 ± 0.2 at 25°. Sterilize in an autoclave using a validated cycle.

Mannitol Salt Agar

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pancreatic Digest of Casein</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Peptic Digest of Animal Tissue</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Beef Extract</td>
<td>1.0 g</td>
</tr>
<tr>
<td>D-Mannitol</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>75.0 g</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0 g</td>
</tr>
<tr>
<td>Phenol Red</td>
<td>0.025 g</td>
</tr>
<tr>
<td>Purified Water</td>
<td>1000 mL</td>
</tr>
</tbody>
</table>

Heat to boiling for 1 minute with shaking. Adjust the pH so that after sterilization it is 7.4 ± 0.2 at 25°. Sterilize in an autoclave using a validated cycle.

Reinforced Medium for Clostridia

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef Extract</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Peptone</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>3.0 g</td>
</tr>
<tr>
<td>Soluble Starch</td>
<td>1.0 g</td>
</tr>
<tr>
<td>Glucose Monohydrate</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Cysteine Hydrochloride</td>
<td>0.5 g</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Sodium Acetate</td>
<td>3.0 g</td>
</tr>
<tr>
<td>Agar</td>
<td>0.5 g</td>
</tr>
<tr>
<td>Purified Water</td>
<td>1000 mL</td>
</tr>
</tbody>
</table>

Hydrate the agar, and dissolve by heating to boiling with continuous stirring. If necessary, adjust the pH so that after sterilization it is about 6.8 ± 0.2 at 25°. Sterilize in an autoclave using a validated cycle.

Columbia Agar

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pancreatic Digest of Casein</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Meat Peptic Digest</td>
<td>5.0 g</td>
</tr>
</tbody>
</table>
Columbia Agar

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart Pancreatic Digest</td>
<td>3.0 g</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Maize Starch</td>
<td>1.0 g</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Agar, according to gelling power</td>
<td>10.0–15.0 g</td>
</tr>
<tr>
<td>Purified Water</td>
<td>1000 mL</td>
</tr>
</tbody>
</table>

Hydrate the agar, and dissolve by heating to boiling with continuous stirring. If necessary, adjust the pH so that after sterilization it is 7.3 ± 0.2 at 25°. Sterilize in an autoclave using a validated cycle. Allow to cool to 45° to 50°; add, where necessary, gentamicin sulfate corresponding to 20 mg of gentamicin base, and pour into Petri dishes.

\[ \text{63) MYCOPLASMA TESTS} \]

**INTRODUCTION**

The genus Mycoplasma represents a group of minute bacteria which have no cell walls. The genus comprises more than 120 species. They are the smallest self-replicating prokaryotic organisms. The cells vary in size and morphology and cannot be Gram stained, but impressions of colonies on solid agar can be stained with methylene blue or equivalent stain. Mycoplasma are parasites and commensals, and some may be pathogenic to a variety of animal and plant hosts. In humans, Mycoplasma are usually surface parasites that colonize the epithelial lining of the respiratory and urogenital tracts. Mycoplasma are common and may cause serious contamination in cell and/or tissue cultures used to generate compendial articles. They may also cause contamination of filtered sterilized soybean casein digest broth. A cell culture infection may persist for an extended period of time without causing apparent cell damage. Infection of cells in a culture can affect nearly every pathway of cell metabolism, including alteration of the cells’ phenotypical characteristics and normal growth. The presence of Mycoplasma species does not always result in turbid growth in cultures or visible alteration of the cells.

Testing for Mycoplasma is a necessary quality control requirement to assure reliably pure biotechnological products and allied materials used to generate these products. This general test chapter describes two methods required to detect Mycoplasma contamination of test articles, tissues and/or cell cultures used to produce test articles, digest broth, or any other material in which Mycoplasma contamination is suspected. These are: (A) the agar and broth media procedure and (B) the indicator cell culture procedure. These tests require careful aseptic technique and suitable laboratory conditions. In order to ensure appropriate testing and interpretation of results, personnel should be properly trained and qualified. A validated nucleic acid amplification technique (NAT) or an enzymatic activity based method may be used to detect Mycoplasma, provided such a method is shown to be comparable to both methods (A) and (B). Alternative methods must be suitably validated. Validation requirements for alternate methods will not be addressed in this chapter.

**CULTURE METHOD**

**Choice of Media**

The test is carried out using a sufficient number of both solid and liquid media to ensure growth in the chosen incubation conditions of small numbers (approximately 100 colony-forming units, cfu; or 100 color-changing units, ccu) of Mycoplasmas that may be present in the test article/material. Liquid media must contain phenol red. The range of media chosen is shown to have satisfactory nutritive properties for at least the microorganisms shown in Quality Control Test Strain Organisms (below). The nutritive properties of each new batch of medium are verified for the appropriate microorganisms in the list. When testing for Mycoplasmas include in each test at least two known Mycoplasma species or strains (listed in Quality Control Test Strain Organisms) as positive controls, one of which should be a dextrose fermenter (i.e., *M. pneumoniae* or equivalent species and strain) and one of which should be an arginine hydrolyzer (i.e., *M. orale* or equivalent species and strain). Only when testing insect cell lines should one include a *Spiroplasma* control strain (e.g., *S. citri* ATCC 29747, *S. melliferum* ATCC 29416, or equivalent species and strains). Additionally, these strains may be a little more fastidious in their nutritional requirements. They require lower incubation temperatures (as do insect cell lines).

**Quality Control Test Strain Organisms**

Positive control cultures should be not more than 15 passages from isolation. Mycoplasma species or strains suitable for use are listed below:

- *Acholeplasma laidlawii* (vaccines and/or cell-derived materials/cultures for human and veterinary use when an antibiotic has been used during production)
- *M. gallisepticum* (when avian material has been used during production or when the vaccine or cell culture is intended for use in poultry)
- *M. hyorhinis* (nonavian veterinary vaccines or cell cultures)
• *M. orale* (vaccines for human and veterinary use)
• *M. pneumoniae* (vaccines or cell banks for human use) or another suitable species of D-glucose fermenter such as *M. fermentans*
• *M. synoviae* (when avian material has been used during production or when the vaccine or cell bank is intended for use in poultry)

The test strains may be field isolates that have undergone a limited number of subcultures (not more than 15), are stored frozen (−20° or lower) or freeze-dried, and are identified as being of the required species by comparison with type cultures, for example, those shown in Table 1.

### Table 1. Type Cultures for Identifying Field Isolates Used as Test Strains

<table>
<thead>
<tr>
<th>Test Organism</th>
<th>NCTC Number</th>
<th>CIP Number</th>
<th>ATCC Number</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. laidlawii</em></td>
<td>NCTC 10116</td>
<td>CIP 75.27</td>
<td>ATCC 23206</td>
</tr>
<tr>
<td><em>M. gallisepticum</em></td>
<td>NCTC 10115</td>
<td>CIP 104967</td>
<td>ATCC 19610</td>
</tr>
<tr>
<td><em>M. fermentans</em></td>
<td>NCTC 10117</td>
<td>CIP 105680</td>
<td>ATCC 19989</td>
</tr>
<tr>
<td><em>M. hyorhinis</em></td>
<td>NCTC 10130</td>
<td>CIP 104968</td>
<td>ATCC 17981</td>
</tr>
<tr>
<td><em>M. orale</em></td>
<td>NCTC 10112</td>
<td>CIP 104969</td>
<td>ATCC 23714</td>
</tr>
<tr>
<td><em>M. pneumoniae</em></td>
<td>NCTC 10119</td>
<td>CIP 103766</td>
<td>ATCC 15531</td>
</tr>
<tr>
<td><em>M. synoviae</em></td>
<td>NCTC 10124</td>
<td>CIP 104970</td>
<td>ATCC 25204</td>
</tr>
</tbody>
</table>

### Incubation Conditions

Incubate liquid media in tightly stoppered containers at 36 ± 1°. Incubate solid media in microaerophilic conditions (hydrogen atmosphere containing < 0.5% oxygen and/or nitrogen containing 5%–10% carbon dioxide in nitrogen). Sufficient humidity should be available to prevent desiccation of the agar surface at 36 ± 1°.

### Nutritive Properties

Carry out the test for nutritive properties for each new batch of medium. Inoculate the chosen media with the appropriate test microorganisms; use not more than 100 cfu per plate containing at least 9 mL of solid media and per 100-mL container of liquid medium; use a separate plate and container for each species of microorganism. Incubate the media and make subcultures from 0.2 mL of liquid medium to solid medium at the specified intervals (see below under Test for Mycoplasma in the Test Article/Material). The solid medium complies with the test if a count within a 0.5-log unit range of the inoculate amount is found for each test microorganism. The liquid medium complies with the test if growth is found on agar plates subcultured from the broth, for at least 1 subculture for each test microorganism. The use of a microscope at 100× or greater may be helpful.

### Inhibitory Substances

The test for inhibitory substances is carried out once for a given product and is repeated whenever there is a change in production method that may affect the detection of Mycoplasma. To demonstrate absence of inhibitory substances, carry out the test for nutritive properties in the presence and absence of the test article/material. If growth of a test microorganism occurs more than 1 subculture sooner in the absence of the test article/material than in its presence, inhibitory substances are present. The same is true if plates directly inoculated with the test article/material are not within a 0.5-log unit range of the number of colonies of those inoculated without the test article/material. In both cases, inhibitory substances must be neutralized or their effect otherwise countered, by an appropriate method, for example, by passage in substrates not containing inhibitors or dilution in a larger volume of medium, before the test. If dilution is used, larger medium volumes may be used or the inoculums’ volume may be divided among several 100-mL flasks. The effectiveness of the neutralization or other process is checked by repeating the test for inhibitory substances after neutralization.

### Test for Mycoplasma in the Test Article/Material

Inoculate no less than 10 mL of the test article/material per 100 mL of each liquid medium. If a significant pH change occurs upon the addition of the test article/material, the liquid medium is restored to its original pH value by the addition of a sterile solution of either sodium hydroxide or hydrochloric acid. Inoculate 0.2 mL of the test article/material on each plate of each solid medium. Incubate liquid media for 20–21 days. Incubate solid media for not less than 14 days, except those plates corresponding to the 20–21 day subculture, which are incubated for 7 days. Concurrently, incubate an uninoculated 100-mL portion of each liquid medium and agar plate, as a negative control. On days 2–4 after inoculation, subculture each liquid medium by inoculating 0.2 mL on at least 1 plate of each solid medium. Repeat the procedure between days 6 and 8, again between days 13 and 15, and again between days 19 and 21 of the test. Observe the liquid media every 2 or 3 days and if a color change occurs, subculture. If a liquid medium shows bacterial or fungal contamination, the test is invalid. The test is valid if at least 1 plate per medium and per inoculation day can be read. Include in the test positive controls prepared by inoculation of not more than 100 cfu of at least 1 test microorganism on agar medium or into broth medium. Where the test for Mycoplasmas is carried out regularly, it is recommended to use the test microorganisms in regular rotation. The test
microorganisms used are those listed under Choice of Media. Incubate broths and plates in a humidified atmosphere with microaerophilic conditions (5%–10% CO₂).

Interpretation of Results

At the end of the prescribed incubation period, examine all inoculated solid media for the presence of Mycoplasma colonies. The product complies with the test if growth of typical Mycoplasma colonies has not occurred. The product does not comply with the test if growth of typical Mycoplasma colonies has occurred on any of the solid media. The test is invalid if 1 or more of the positive controls do not show growth of Mycoplasmas on at least 1 subculture plate. The test is invalid if 1 or more of the negative controls show growth of Mycoplasmas. If suspect colonies are observed, use a suitable validated method to determine whether they are due to Mycoplasmas.

Recommended Solutions and Media for the Culture Method

[SPECIAL NOTE—This section is provided for information.]

**SOLUTIONS**

**Beef Heart Infusion Broth**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef heart (for preparation of the infusion)</td>
<td>500 g</td>
</tr>
<tr>
<td>Peptone</td>
<td>10 g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>to 1000 mL</td>
</tr>
</tbody>
</table>

**Essential Vitamins**

<table>
<thead>
<tr>
<th>Vitamin</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biotin</td>
<td>100 mg</td>
</tr>
<tr>
<td>Calcium pantothenate</td>
<td>100 mg</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>100 mg</td>
</tr>
<tr>
<td>Folic acid</td>
<td>100 mg</td>
</tr>
<tr>
<td>1-inositol</td>
<td>200 mg</td>
</tr>
<tr>
<td>Nicotinamide</td>
<td>100 mg</td>
</tr>
<tr>
<td>Pyridoxal hydrochloride</td>
<td>100 mg</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>10 mg</td>
</tr>
<tr>
<td>Thiamine hydrochloride</td>
<td>100 mg</td>
</tr>
<tr>
<td>Distilled water</td>
<td>to 1000 mL</td>
</tr>
</tbody>
</table>

**Agar, Purified**

A highly refined agar for use in microbiology and immunology, prepared by an ion-exchange procedure that results in a product having superior purity, clarity, and gel strength. It contains the following ingredients:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>12.2%</td>
</tr>
<tr>
<td>Ash</td>
<td>1.5%</td>
</tr>
<tr>
<td>Acid-insoluble ash</td>
<td>0.2%</td>
</tr>
<tr>
<td>Chlorine</td>
<td>0</td>
</tr>
<tr>
<td>Phosphate (calculated as P₂O₅)</td>
<td>0.3%</td>
</tr>
<tr>
<td>Total nitrogen</td>
<td>0.3%</td>
</tr>
<tr>
<td>Copper</td>
<td>8 ppm</td>
</tr>
<tr>
<td>Iron</td>
<td>170 ppm</td>
</tr>
<tr>
<td>Calcium</td>
<td>0.28%</td>
</tr>
<tr>
<td>Magnesium</td>
<td>0.32%</td>
</tr>
</tbody>
</table>

**Hanks’ Balanced Salt Solution (modified)**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium chloride</td>
<td>6.4 g</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>0.32 g</td>
</tr>
<tr>
<td>Magnesium sulphate heptahydrate</td>
<td>0.08 g</td>
</tr>
<tr>
<td>Magnesium chloride hexahydrate</td>
<td>0.08 g</td>
</tr>
<tr>
<td>Calcium chloride, anhydrous</td>
<td>0.112 g</td>
</tr>
</tbody>
</table>
Hanks' Balanced Salt Solution (modified) (continued)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disodium hydrogen phosphate dihydrate</td>
<td>0.0596 g</td>
</tr>
<tr>
<td>Potassium dihydrogen phosphate, anhydrous</td>
<td>0.048 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>to 800 mL</td>
</tr>
</tbody>
</table>

Brain Heart Infusion

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calf-brain infusion</td>
<td>200 g</td>
</tr>
<tr>
<td>Beef-heart infusion</td>
<td>250 g</td>
</tr>
<tr>
<td>Proteose peptone</td>
<td>10 g</td>
</tr>
<tr>
<td>Glucose monohydrate</td>
<td>2 g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5 g</td>
</tr>
<tr>
<td>Disodium hydrogen phosphate, anhydrous</td>
<td>2.5 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>to 1000 mL</td>
</tr>
</tbody>
</table>

PPLO Broth

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef-heart infusion</td>
<td>50 g</td>
</tr>
<tr>
<td>Peptone</td>
<td>10 g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>to 1000 mL</td>
</tr>
</tbody>
</table>

MEDIA

The following media are recommended. Other media may be used, provided they meet the criteria given in the sections Choice of Culture Media, Incubation Conditions, Nutritive Properties, and Inhibitory Substances.

Hayflick Media (Recommended for the general detection of Mycoplasmas)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef heart infusion broth</td>
<td>90.0 mL</td>
</tr>
<tr>
<td>Horse serum (unheated)</td>
<td>20.0 mL</td>
</tr>
<tr>
<td>Yeast extract (250 g/L) (fresh yeast extract is recommended)</td>
<td>10.0 mL</td>
</tr>
<tr>
<td>Phenol red (0.6 g/L solution)</td>
<td>5.0 mL</td>
</tr>
<tr>
<td>Penicillin (20,000 IU/mL)</td>
<td>0.25 mL</td>
</tr>
<tr>
<td>Deoxyribonucleic acid (2 g/L solution)</td>
<td>1.2 mL</td>
</tr>
<tr>
<td>Adjust to a pH of 7.8</td>
<td></td>
</tr>
</tbody>
</table>

Solid Medium

Prepare as described above replacing beef heart infusion broth by beef heart infusion agar containing 15 g/L of agar.

Frey Media (Recommended for the detection of *M. synoviae*)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef heart infusion broth</td>
<td>90.0 mL</td>
</tr>
<tr>
<td>Essential vitamins</td>
<td>0.025 mL</td>
</tr>
<tr>
<td>Glucose monohydrate (500 g/L solution)</td>
<td>2.0 mL</td>
</tr>
<tr>
<td>Swine serum (inactivated at 56° for 30 min)</td>
<td>12.0 mL</td>
</tr>
<tr>
<td>β-Nicotinamide adenine dinucleotide (10 g/L solution)</td>
<td>1.0 mL</td>
</tr>
<tr>
<td>Cysteine hydrochloride (10 g/L solution)</td>
<td>1.0 mL</td>
</tr>
<tr>
<td>Phenol red (0.6 g/L solution)</td>
<td>5.0 mL</td>
</tr>
<tr>
<td>Penicillin (20,000 IU/mL)</td>
<td>0.25 mL</td>
</tr>
<tr>
<td>Mix the solutions of β-nicotinamide adenine dinucleotide and cysteine hydrochloride and after 10 min add to the other ingredients. Adjust to a pH of 7.8.</td>
<td></td>
</tr>
</tbody>
</table>

Solid Medium

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef heart infusion broth</td>
<td>90.0 mL</td>
</tr>
<tr>
<td>Agar, purified</td>
<td>1.4 g</td>
</tr>
</tbody>
</table>
**Frey Media (Recommended for the detection of M. synoviae) (continued)**

Adjust to pH 7.8, sterilize by autoclaving then add:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Essential vitamins</td>
<td>0.025 mL</td>
</tr>
<tr>
<td>Glucose monohydrate (500 g/L solution)</td>
<td>2.0 mL</td>
</tr>
<tr>
<td>Swine serum (unheated)</td>
<td>12.0 mL</td>
</tr>
<tr>
<td>β-Nicotinamide adenine dinucleotide (10 g/L solution)</td>
<td>1.0 mL</td>
</tr>
<tr>
<td>Cysteine hydrochloride (10 g/L solution)</td>
<td>1.0 mL</td>
</tr>
<tr>
<td>Phenol red (0.6 g/L solution)</td>
<td>5.0 mL</td>
</tr>
<tr>
<td>Penicillin (20,000 IU/mL)</td>
<td>0.25 mL</td>
</tr>
</tbody>
</table>

**Friis Media (Recommended for the detection of nonavian Mycoplasmas)**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hanks’ balanced salt solution (modified)</td>
<td>800 mL</td>
</tr>
<tr>
<td>Distilled water</td>
<td>67 mL</td>
</tr>
<tr>
<td>Brain heart infusion</td>
<td>135 mL</td>
</tr>
<tr>
<td>PPLO Broth</td>
<td>248 mL</td>
</tr>
<tr>
<td>Yeast extract (1.70 g/L)</td>
<td>60 mL</td>
</tr>
<tr>
<td>Bactracin</td>
<td>250 mg</td>
</tr>
<tr>
<td>Meticillin</td>
<td>250 mg</td>
</tr>
<tr>
<td>Phenol red (5 g/L)</td>
<td>4.5 mL</td>
</tr>
<tr>
<td>Horse serum</td>
<td>165 mL</td>
</tr>
<tr>
<td>Swine serum</td>
<td>165 mL</td>
</tr>
<tr>
<td>Adjust to a pH of 7.40–7.45</td>
<td></td>
</tr>
</tbody>
</table>

**Solid Medium**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hanks’ balanced salt solution (modified)</td>
<td>200 mL</td>
</tr>
<tr>
<td>DEAE-dextran</td>
<td>200 mg</td>
</tr>
<tr>
<td>Agar, purified</td>
<td>15.65 g</td>
</tr>
</tbody>
</table>

Mix well and sterilize by autoclaving. Cool to 100°. Add to 1740 mL of Liquid Medium as described above.

**INDICATOR CELL CULTURE METHOD**

Cell cultures are stained with a fluorescent dye that binds to DNA. Mycoplasmas are detected by their characteristic particulate or filamentous pattern of fluorescence on the cell surface and, if contamination is heavy, in surrounding areas. Mitochondria in the cytoplasm may be stained but are readily distinguished from Mycoplasmas. For viral suspensions, if the interpretation of results is affected by marked cytopathic effects, neutralize the virus using a specific antiserum that has no inhibitory effects on Mycoplasmas, or use a cell culture substrate that does not allow growth of the virus. To demonstrate the absence of inhibitory effects of serum, carry out the positive control tests in the presence and absence of the antiserum.

**Verification of the Substrate**

Use Vero cells or equivalent cell culture (for example, the production cell line) that is equivalent in effectiveness for detecting Mycoplasmas. Test the effectiveness of the cells to be used by applying the procedure shown below and inoculating not more than 100 cfu or ccu microorganisms of suitable reference strains of *M. hyorhinis* and *M. orale*. The cells are suitable if both reference strains are detected. The indicator cells must be subcultured without an antibiotic before use in the test.

**Test Method**

[NOTE—The following is provided for information.]

**SOLUTIONS**

**Phosphate Buffered Saline**

2.0 M Monobasic Potassium Phosphate—Dissolve 13.61 g of anhydrous monobasic potassium phosphate in 50 mL of water.

2.0 M Dibasic Potassium Phosphate—Dissolve 17.42 g of anhydrous dibasic potassium phosphate in 50 mL of water.

Phosphate Buffered Saline Solution (pH 7.4)—Combine 3.6 mL of 2.0 M Monobasic Potassium Phosphate, 16.4 mL of 2.0 M Dibasic Potassium Phosphate, 8 g of sodium chloride, and 1 L of water. Mix thoroughly. Adjust the pH if necessary.
**Bisbenzimide Stock Solution**—Dissolve 5 mg of bisbenzimide in water, and dilute with the same solvent to 100 mL. Store in the dark.

**Bisbenzimide Working Solution**—Immediately before use, dilute 100 µL of Bisbenzimide Stock Solution with Phosphate Buffered Saline Solution (pH 7.4) to 100 mL.

**Phosphate-Citrate Buffer Solution pH 5.5**—Mix 56.85 mL of a 28.4-g/L solution of anhydrous disodium hydrogen phosphate and 43.15 mL of a 21-g/L solution of citric acid.

**METHOD**

1. Seed the indicator cell culture at a suitable density (for example, $2 \times 10^4$ to $2 \times 10^5$ cells/mL, $4 \times 10^3$ to $2.5 \times 10^4$ cells/cm$^2$) that will yield confluence after 3 days of growth. Inoculate 1 mL of the product to be examined into the cell culture vessel, and incubate at 36 ± 1°C.

2. After at least 3 days of incubation, when the cells have grown to confluence, make a subculture on cover slips in suitable containers or on some other surface (for example, chambered slides) suitable for the test procedure. Seed the cells at low density so that they reach 50% confluence after 3–5 days of incubation. Complete confluence impairs visualization of Mycoplasmas after staining and must be avoided.

3. Remove the medium and rinse the indicator cells with phosphate buffered saline, pH 7.4, then add a suitable fixing solution (a freshly prepared mixture of 1 volume of acetic acid, glacial, TS and 3 volumes of methanol, is suitable when bisbenzimide is used for staining).

4. Remove the fixing solution and wash the cells with sterile Purified Water. Dry the slides completely if they are to be stained more than 1 hour later (particular care is needed for staining of slides after drying owing to artifacts that may be produced).

5. Add a suitable DNA stain and allow standing for a suitable time (bisbenzimide working solution and a standing time of 10 minutes are suitable).

6. Remove the stain and rinse the monolayer with Purified Water.

7. Mount each coverslip, where applicable (a mixture of equal volumes of glycerol and Phosphate-Citrate Buffer Solution pH 5.5 is suitable for mounting). Examine by fluorescence (for bisbenzimide stain a 330 nm/380 nm excitation filter and an LP 440 nm barrier filter are suitable) at 400× magnification or greater.

8. Compare the microscopic appearance of the test cultures with that of the negative and positive controls, examining for extranuclear fluorescence. Mycoplasmas produce pinpoints or filaments over the indicator cell cytoplasm. They may also produce pinpoints and filaments in the intercellular spaces. Multiple microscopic fields are examined according to the protocol established during validation.

**Interpretation of Results**

The product to be examined complies with the test if fluorescence typical of Mycoplasmas is not present. The test is invalid if the positive controls do not show fluorescence typical of Mycoplasmas. The test is invalid if the negative controls show fluorescence typical of Mycoplasmas.

**〈71〉 STERILITY TESTS**

*Portions of this general chapter have been harmonized with the corresponding texts of the European Pharmacopeia and/or the Japanese Pharmacopeia. Those portions that are not harmonized are marked with symbols (◆) to specify this fact.*

These Pharmacopoeial procedures are not by themselves designed to ensure that a batch of product is sterile or has been sterilized. This is accomplished primarily by validation of the sterilization process or of the aseptic processing procedures. The test is applied to substances, preparations, or articles which, according to the Pharmacopeia, are required to be sterile. However, a satisfactory result only indicates that no contaminating microorganism has been found in the sample examined under the conditions of the test.

**PRECAUTIONS AGAINST MICROBIAL CONTAMINATION**

The test for sterility is carried out under aseptic conditions. In order to achieve such conditions, the test environment has to be adapted to the way in which the sterility test is performed. The precautions taken to avoid contamination are such that they do not affect any microorganisms that are to be revealed in the test. The working conditions in which the tests are performed are monitored regularly by appropriate sampling of the working area and by carrying out appropriate controls.

**CULTURE MEDIA AND INCUBATION TEMPERATURES**

Media for the test may be prepared as described below or equivalent commercial media may be used provided that they comply with the requirements of the Growth Promotion Test of Aerobes, Anaerobes, and Fungi. The following culture media have been found to be suitable for the test for sterility. Fluid Thioglycollate Medium is primarily intended for the culture of anaerobic bacteria. However, it will also detect aerobic bacteria. Soybean–Casein Digest Medium is suitable for the culture of both fungi and aerobic bacteria.
**Fluid Thioglycollate Medium**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Cystine</td>
<td>0.5 g</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>2.5 g</td>
</tr>
<tr>
<td>Dextrose Monohydrate/Anhydrous</td>
<td>5.5/5.0 g</td>
</tr>
<tr>
<td>Agar</td>
<td>0.75 g</td>
</tr>
<tr>
<td>Yeast Extract (water-soluble)</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Pancreatic Digest of Casein</td>
<td>15.0 g</td>
</tr>
<tr>
<td>Sodium Thioglycollate or Thioglycolic Acid</td>
<td>0.5 g/0.3 mL</td>
</tr>
<tr>
<td>Resazurin Sodium Solution (1 in 1000), freshly prepared</td>
<td>1.0 mL</td>
</tr>
<tr>
<td>Purified Water</td>
<td>1000 mL</td>
</tr>
</tbody>
</table>

pH after sterilization: 7.1±0.2.

Mix the L-cystine, agar, sodium chloride, dextrose, yeast extract, and pancreatic digest of casein with the purified water, and heat until solution is effected. Dissolve the sodium thioglycollate or thioglycolic acid in the solution and, if necessary, add 1 N sodium hydroxide so that, after sterilization, the solution will have a pH of 7.1 ± 0.2. If filtration is necessary, heat the solution again without boiling, and filter while hot through moistened filter paper. Add the resazurin sodium solution, mix, and place the medium in suitable vessels that provide a ratio of surface to depth of medium such that not more than the upper half of the medium has undergone a color change indicative of oxygen uptake at the end of the incubation period. Sterilize using a validated process. If the medium is stored, store at a temperature between 2° and 25° in a sterile, airtight container. If more than the upper one-third of the medium has acquired a pink color, the medium may be restored once by heating the containers in a water-bath or in free-flowing steam until the pink color disappears and by cooling quickly, taking care to prevent the introduction of nonsterile air into the container. Do not use the medium for a longer storage period than has been validated.

**Fluid Thioglycollate Medium** is to be incubated at 30°–35°. For products containing a mercurial preservative that cannot be tested by the membrane filtration method, **Fluid Thioglycollate Medium** incubated at 20°–25° may be used instead of **Soybean–Casein Digest Medium** provided that it has been validated as described in *Growth Promotion Test of Aerobes, Anaerobes, and Fungi*. Where prescribed or justified and authorized, the following alternative thioglycollate medium might be used.

Prepare a mixture having the same composition as that of the **Fluid Thioglycollate Medium**, but omitting the agar and the resazurin sodium solution. Sterilize as directed above. The pH after sterilization is 7.1 ± 0.2. Heat in a water bath prior to use and incubate at 30°–35° under anaerobic conditions.

**Soybean–Casein Digest Medium**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pancreatic Digest of Casein</td>
<td>17.0 g</td>
</tr>
<tr>
<td>Papaic Digest of Soybean Meal</td>
<td>3.0 g</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Dibasic Potassium Phosphate</td>
<td>2.5 g</td>
</tr>
<tr>
<td>Dextrose Monohydrate/Anhydrous</td>
<td>2.5/2.3 g</td>
</tr>
<tr>
<td>Purified Water</td>
<td>1000 mL</td>
</tr>
</tbody>
</table>

pH after sterilization: 7.3±0.2.

Dissolve the solids in the Purified Water, heating slightly to effect a solution. Cool the solution to room temperature, and adjust the pH with 1 N sodium hydroxide so that, after sterilization, it will have a pH of 7.3 ± 0.2. Filter, if necessary to clarify, dispense into suitable containers, and sterilize using a validated procedure. Store at a temperature between 2° and 25° in a sterile well-closed container, unless it is intended for immediate use. Do not use the medium for a longer storage period than has been validated.

**Soybean–Casein Digest Medium** is to be incubated at 22.5 ± 2.5°.

**Media for Penicillins or Cephalosporins**

Where sterility test media are to be used in the **Direct Inoculation of the Culture Medium** method under **Test for Sterility of the Product to be Examined**, modify the preparation of **Fluid Thioglycollate Medium** and the **Soybean–Casein Digest Medium** as follows. To the containers of each medium, transfer aseptically a quantity of β-lactamase sufficient to inactivate the amount of antibiotic in the specimen under test. Determine the quantity of β-lactamase required to inactivate the antibiotic by using a β-lactamase preparation that has been assayed previously for its penicillin- or cephalosporin-inactivating power. [NOTE—Supplemented β-lactamase media can also be used in the membrane filtration test.]

Alternatively (in an area completely separate from that used for sterility testing), confirm that an appropriate amount of β-lactamase is incorporated into the medium, following either method under **Method Suitability Test**, using less than 100 colony-forming units (cfu) of *Staphylococcus aureus* (see Table 1) as the challenge. Typical microbial growth of the inoculated culture must be observed as a confirmation that the β-lactamase concentration is appropriate.*

*Not official text. Please refer to the currently official version of the applicable USP–NF or FCC standard for compliance purposes.*

Published on March 26, 2020
### Table 1. Strains of the Test Microorganisms Suitable for Use in the Growth Promotion Test and the Method Suitability Test

<table>
<thead>
<tr>
<th>Aerobic bacteria</th>
<th>ATCC</th>
<th>CIP</th>
<th>NCTC</th>
<th>NCIMB</th>
<th>NBRC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staphylococcus aureus</td>
<td>6538</td>
<td></td>
<td>10788</td>
<td>9518</td>
<td>13276</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>6633</td>
<td></td>
<td>52.62</td>
<td>8054</td>
<td>3134</td>
</tr>
<tr>
<td>Pseudomonas aeruginosan*1</td>
<td>9027</td>
<td></td>
<td>8626</td>
<td>82.118</td>
<td>13275</td>
</tr>
<tr>
<td>Anaerobic bacterium</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clostridium sporogenes*2</td>
<td>19404</td>
<td>79.3</td>
<td>532</td>
<td>11437</td>
<td>14293</td>
</tr>
<tr>
<td>Fungi</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Candida albicans</td>
<td>10231</td>
<td></td>
<td>48.72</td>
<td>3179</td>
<td>1594</td>
</tr>
<tr>
<td>Aspergillus brasiliensis* (Aspergillus Niger)</td>
<td>16404</td>
<td>1431.83</td>
<td></td>
<td>149007</td>
<td>9455</td>
</tr>
</tbody>
</table>

*1 An alternative microorganism is *Kocuria rhizophila* (*Micrococcus luteus*) ATCC 9341.

*2 An alternative to *Clostridium sporogenes,* when a nonspore-forming microorganism is desired, is *Bacteroides vulgatus* (ATCC 8482).

The media used comply with the following tests, carried out before, or in parallel, with the test on the product to be examined.

**Sterility**

Incubate portions of the media for 14 days. No growth of microorganisms occurs.

**Growth Promotion Test of Aerobes, Anaerobes, and Fungi**

Test each lot of ready-prepared medium and each batch of medium prepared either from dehydrated medium or from ingredients. Suitable strains of microorganisms are indicated in Table 1. Inoculate portions of Fluid Thioglycollate Medium with a small number (not more than 100 cfu) of the following microorganisms, using a separate portion of medium for each of the following species of microorganism: *Clostridium sporogenes,* *Pseudomonas aeruginosan,* and *Staphylococcus aureus.* Inoculate portions of alternative thioglycollate medium with a small number (not more than 100 cfu) of *Clostridium sporogenes,* Inoculate portions of Soybean–Casein Digest Medium with a small number (not more than 100 cfu) of the following microorganisms, using a separate portion of medium for each of the following species of microorganism: *Aspergillus brasiliensis,* *Bacillus subtilis,* and *Candida albicans.* Incubate for not more than 3 days in the case of bacteria and not more than 5 days in the case of fungi. Seed lot culture maintenance techniques (seed-lot systems) are used so that the viable microorganisms used for inoculation are not more than five passages removed from the original master seed-lot.

The media are suitable if a clearly visible growth of the microorganisms occurs.

**‘DILUTING AND RINSING FLUIDS FOR MEMBRANE FILTRATION**

**Fluid A**

**PREPARATION**

Dissolve 1 g of peptic digest of animal tissue in water to make 1 L, filter or centrifuge to clarify, if necessary, and adjust to a pH of 7.1 ± 0.2. Dispense into containers, and sterilize using a validated process.

**PREPARATION FOR PENICILLINS OR CEPHALOSPORINS**

Aseptically add to the above Preparation, if necessary, a quantity of sterile β-lactamase sufficient to inactivate any residual antibiotic activity on the membranes after the solution of the test specimen has been filtered (see Media for Penicillins or Cephalosporins).

**Fluid D**

To each L of Fluid A add 1 mL of polysorbate 80, adjust to a pH of 7.1 ± 0.2, dispense into containers, and sterilize using a validated process. Use this fluid for articles containing lecithin or oil, or for devices labeled as “sterile pathway.”

**Fluid K**

Dissolve 5.0 g of peptic digest of animal tissue, 3.0 g of beef extract, and 10.0 g of polysorbate 80 in water to make 1 L. Adjust the pH to obtain, after sterilization, a pH of 6.9 ± 0.2. Dispense into containers, and sterilize using a validated process.

Published on March 26, 2020
METHOD SUITABILITY TEST

Carry out a test as described below under Test for Sterility of the Product to be Examined using exactly the same methods, except for the following modifications.

Membrane Filtration

After transferring the content of the container or containers to be tested to the membrane, add an inoculum of a small number of viable microorganisms (not more than 100 cfu) to the final portion of sterile diluent used to rinse the filter.

Direct Inoculation

After transferring the contents of the container or containers to be tested (for catgut and other surgical sutures for veterinary use: strands) to the culture medium, add an inoculum of a small number of viable microorganisms (not more than 100 cfu) to the medium.

In both cases use the same microorganisms as those described above under Growth Promotion Test of Aerobes, Anaerobes, and Fungi. Perform a growth promotion test as a positive control. Incubate all the containers containing medium for not more than 5 days.

If clearly visible growth of microorganisms is obtained after the incubation, visually comparable to that in the control vessel without product, either the product possesses no antimicrobial activity under the conditions of the test or such activity has been satisfactorily eliminated. The test for sterility may then be carried out without further modification.

If clearly visible growth is not obtained in the presence of the product to be tested, visually comparable to that in the control vessels without product, the product possesses antimicrobial activity that has not been satisfactorily eliminated under the conditions of the test. Modify the conditions in order to eliminate the antimicrobial activity, and repeat the Method Suitability Test.

This method suitability is performed (a) when the test for sterility has to be carried out on a new product; and (b) whenever there is a change in the experimental conditions of the test. The method suitability may be performed simultaneously with the Test for Sterility of the Product to be Examined.

TEST FOR STERILITY OF THE PRODUCT TO BE EXAMINED

*Number of Articles to Be Tested

Unless otherwise specified elsewhere in this chapter or in the individual monograph, test the number of articles specified in Table 3. If the contents of each article are of sufficient quantity (see Table 2), they may be divided so that equal appropriate portions are added to each of the specified media. [NOTE—Perform sterility testing employing two or more of the specified media.] If each article does not contain sufficient quantities for each medium, use twice the number of articles indicated in Table 3.

<table>
<thead>
<tr>
<th>Table 2. Minimum Quantity to be Used for Each Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Quantity per Container</strong></td>
</tr>
<tr>
<td><strong>Liquids</strong></td>
</tr>
<tr>
<td>Less than 1 mL</td>
</tr>
<tr>
<td>1–40 mL</td>
</tr>
<tr>
<td>Greater than 40 mL, and not greater than 100 mL</td>
</tr>
<tr>
<td>Greater than 100 mL</td>
</tr>
<tr>
<td>Antibiotic liquids</td>
</tr>
<tr>
<td>Insoluble preparations, creams, and ointments to be suspended or emulsified</td>
</tr>
<tr>
<td><strong>Solids</strong></td>
</tr>
<tr>
<td>Less than 50 mg</td>
</tr>
<tr>
<td>50 mg or more, but less than 300 mg</td>
</tr>
<tr>
<td>300 mg–5 g</td>
</tr>
<tr>
<td>Greater than 5 g</td>
</tr>
<tr>
<td>Catgut and other surgical sutures for veterinary use</td>
</tr>
<tr>
<td>*Surgical dressing/cotton/gauze (in packages)</td>
</tr>
<tr>
<td>Sutures and other individually packaged single-use material</td>
</tr>
<tr>
<td>Other medical devices</td>
</tr>
</tbody>
</table>
Table 3. Minimum Number of Articles to be Tested in Relation to the Number of Articles in the Batch

<table>
<thead>
<tr>
<th>Number of Items in the Batch*</th>
<th>Minimum Number of Items to be Tested for Each Medium (unless otherwise justified and authorized)**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parenteral preparations</td>
<td></td>
</tr>
<tr>
<td>Not more than 100 containers</td>
<td>10% or 4 containers, whichever is the greater</td>
</tr>
<tr>
<td>More than 100 but not more than 500 containers</td>
<td>10 containers</td>
</tr>
<tr>
<td>More than 500 containers</td>
<td>2% or 20 containers, whichever is less</td>
</tr>
<tr>
<td>* For large-volume parenterals</td>
<td>2% or 10 containers, whichever is less</td>
</tr>
<tr>
<td>Antibiotic solids</td>
<td></td>
</tr>
<tr>
<td>Pharmacy bulk packages (&lt;5 g)</td>
<td>20 containers</td>
</tr>
<tr>
<td>Pharmacy bulk packages (≥5 g)</td>
<td>6 containers</td>
</tr>
<tr>
<td>Bulks and blends</td>
<td>See Bulk solid products</td>
</tr>
<tr>
<td>◆ For large-volume parenterals</td>
<td>2% or 10 containers, whichever is less</td>
</tr>
<tr>
<td>Ophthalmic and other noninjectable preparations</td>
<td></td>
</tr>
<tr>
<td>Not more than 200 containers</td>
<td>5% or 2 containers, whichever is the greater</td>
</tr>
<tr>
<td>More than 200 containers</td>
<td>10 containers</td>
</tr>
<tr>
<td>If the product is presented in the form of single-dose containers, apply the scheme shown above for preparations for parenteral use.</td>
<td></td>
</tr>
<tr>
<td>Catgut and other surgical sutures for veterinary use</td>
<td>2% or 5 packages, whichever is the greater, up to a maximum total of 20 packages</td>
</tr>
<tr>
<td>*Not more than 100 articles</td>
<td>10% or 4 articles, whichever is greater</td>
</tr>
<tr>
<td>More than 100, but not more than 500 articles</td>
<td>10 articles</td>
</tr>
<tr>
<td>More than 500 articles</td>
<td>2% or 20 articles, whichever is less,</td>
</tr>
<tr>
<td>Bulk solid products</td>
<td></td>
</tr>
<tr>
<td>Up to 4 containers</td>
<td>Each container</td>
</tr>
<tr>
<td>More than 4 containers, but not more than 50 containers</td>
<td>20% or 4 containers, whichever is greater</td>
</tr>
<tr>
<td>More than 50 containers</td>
<td>2% or 10 containers, whichever is greater</td>
</tr>
</tbody>
</table>

* If the batch size is unknown, use the maximum number of items prescribed.
** If the contents of one container are enough to inoculate the two media, this column gives the number of containers needed for both the media together.

The test may be carried out using the technique of Membrane Filtration or by Direct Inoculation of the Culture Medium with the product to be examined. Appropriate negative controls are included. The technique of membrane filtration is used whenever the nature of the product permits; that is, for filterable aqueous preparations, for alcoholic or oily preparations, and for preparations miscible with, or soluble in, aqueous or oily solvents, provided these solvents do not have an antimicrobial effect in the conditions of the test.

Membrane Filtration

Use membrane filters having a nominal pore size not greater than 0.45 µm, in which the effectiveness to retain microorganisms has been established. Cellulose nitrate filters, for example, are used for aqueous, oily, and weakly alcoholic solutions; and cellulose acetate filters, for example, are used for strongly alcoholic solutions. Specially adapted filters may be needed for certain products (e.g., for antibiotics).

The technique described below assumes that membranes about 50 mm in diameter will be used. If filters of a different diameter are used, the volumes of the dilutions and the washings should be adjusted accordingly. The filtration apparatus and membrane are sterilized by appropriate means. The apparatus is designed so that the solution to be examined can be introduced and filtered under aseptic conditions; it permits the aseptic removal of the membrane for transfer to the medium, or it is suitable for carrying out the incubation after adding the medium to the apparatus itself.

AQUEOUS SOLUTIONS

If appropriate, transfer a small quantity of a suitable, sterile diluent such as *Fluid A (see Diluting and Rinsing Fluids for Membrane Filtration), onto the membrane in the apparatus and filter. The diluent may contain suitable neutralizing substances and/or appropriate inactivating substances, for example, in the case of antibiotics.

Transfer the contents of the container or containers to be tested to the membrane or membranes, if necessary, after diluting to the volume used in the Method Suitability Test with the chosen sterile diluent, but using not less than the quantities of the product to be examined prescribed in Tables 2 and 3. Filter immediately. If the product has antimicrobial properties, wash the membrane not less than three times by filtering through it each time the volume of the chosen sterile diluent used in the Method.
Suitability Test. Do not exceed a washing cycle of five times 100 mL per filter, even if during method suitability it has been demonstrated that such a cycle does not fully eliminate the antimicrobial activity. Transfer the whole membrane to the culture medium or cut it aseptically into two equal parts, and transfer one half to each of two suitable media. Use the same volume of each medium as in the Method Suitability Test. Alternatively, transfer the medium onto the membrane in the apparatus. Incubate the media for not less than 14 days.

SOLUBLE SOLIDS

Use for each medium not less than the quantity prescribed in Tables 2 and 3 of the product dissolved in a suitable solvent, such as the solvent provided with the preparation, Sterile Water for Injection, sterile saline, or a suitable sterile solution such as Fluid A (Diluting and Rinsing Fluids for Membrane Filtration), and proceed with the test as described above for Aqueous Solutions using a membrane appropriate to the chosen solvent.

OILS and OILY SOLUTIONS

Use for each medium not less than the quantity of the product prescribed in Tables 2 and 3. Oils and oily solutions of sufficiently low viscosity may be filtered without dilution through a dry membrane. Viscous oils may be diluted as necessary with a suitable sterile diluent such as isopropyl myristate shown not to have antimicrobial activity in the conditions of the test. Allow the oil to penetrate the membrane by its own weight, and then filter, applying the pressure or suction gradually. Wash whichever applies. If necessary, excess diluent can be added to aid in the constitution and filtration of the constituted solution

Use for each medium not less than the quantity of the product prescribed in Tables 2 and 3. Oil solutions may be diluted by heating, if necessary, to not more than 46°. In exceptional cases it may be necessary to heat to not more than 44°. Filter as rapidly as possible, and proceed as described above for Aqueous Solutions.

OINTMENTS and CREAMS

Use for each medium not less than the quantities of the product prescribed in Tables 2 and 3. Ointments in a fatty base and emulsions of the water-in-oil type may be diluted to 1% in isopropyl myristate as described above, by heating, if necessary, to not more than 46°. In exceptional cases it may be necessary to heat to not more than 44°. Filter as rapidly as possible, and proceed as described above for Oils and Oily Solutions.

PREPARED SYRINGES

For prefilled syringes without attached sterile needles, expel the contents of each syringe into one or two separate membrane filter funnels or into separate pooling vessels prior to transfer. If a separate sterile needle is attached, directly expel the syringe contents as indicated above, and proceed as directed for Aqueous Solutions. Test the sterility of the needle, using Direct Inoculation under Method Suitability Test.

SOLIDS FOR INJECTION OTHER THAN ANTIBIOTICS

Constitute the test articles as directed on the label, and proceed as directed for Aqueous Solutions or Oils and Oily Solutions, whichever applies. [NOTE—If necessary, excess diluent can be added to aid in the constitution and filtration of the constituted test article.]

ANTIBIOTIC SOLIDS FOR INJECTION

Pharmacy Bulk Packages, <5 g—From each of 20 containers, aseptically transfer about 300 mg of solids, into a sterile 500-mL conical flask, dissolve in about 200 mL of Fluid A (see Diluting and Rinsing Fluids for Membrane Filtration), and mix; or constitute, as directed in the labeling, each of 20 containers and transfer a quantity of liquid or suspension, equivalent to about 300 mg of solids, into a sterile 500-mL conical flask, dissolve in about 200 mL of Fluid A, and mix. Proceed as directed for Aqueous Solutions or Oils and Oily Solutions, whichever applies.

Pharmacy Bulk Packages, ≥5 g—From each of 6 containers, aseptically transfer about 1 g of solids into a sterile 500-mL conical flask, dissolve in about 200 mL of Fluid A, and mix; or constitute, as directed in the labeling, each of 6 containers and transfer a quantity of liquid, equivalent to about 1 g of solids, into a sterile 500-mL conical flask, dissolve in about 200 mL of Fluid A, and mix. Proceed as directed for Aqueous Solutions.

ANTIBIOTIC SOLIDS, BULKS, and BLENDS

Aseptically remove a sufficient quantity of solids from the appropriate amount of containers (see Table 2), mix to obtain a composite, equivalent to about 6 g of solids, and transfer to a sterile 500-mL conical flask. Dissolve in about 200 mL of Fluid A, and mix. Proceed as directed for Aqueous Solutions.

STERILE AEROSOL PRODUCTS

For fluid products in pressurized aerosol form, freeze the containers in an alcohol-dry ice mixture at least at –20° for about 1 hour. If feasible, allow the propellant to escape before aseptically opening the container, and transfer the contents to a sterile pooling vessel. Add 100 mL of Fluid D to the pooling vessel, and mix gently. Proceed as directed for Aqueous Solutions or Oils and Oily Solutions, whichever applies.
DEVICES WITH PATHWAYS LABELED STERILE

Aseptically pass not less than 10 pathway volumes of Fluid D through each device tested. Collect the fluids in an appropriate sterile vessel, and proceed as directed for Aqueous Solutions or Oils and Oily Solutions, whichever applies.

In the case of sterile, empty syringes, draw sterile diluent into the barrel through the sterile needle, if attached, or through a sterile needle attached for the purpose of the test, and express the contents into a sterile pooling vessel. Proceed as directed above.

Direct Inoculation of the Culture Medium

Transfer the quantity of the preparation to be examined prescribed in Tables 2 and 3 directly into the culture medium so that the volume of the product is not more than 10% of the volume of the medium, unless otherwise prescribed.

If the product to be examined has antimicrobial activity, carry out the test after neutralizing this with a suitable neutralizing substance or by dilution in a sufficient quantity of culture medium. When it is necessary to use a large volume of the product, it may be preferable to use a concentrated culture medium prepared in such a way that it takes into account the subsequent dilution. Where appropriate, the concentrated medium may be added directly to the product in its container.

OILY LIQUIDS

Use media to which have been added a suitable emulsifying agent at a concentration shown to be appropriate in the Method Suitability Test, for example polysorbate 80 at a concentration of 10 g per L.

OINTMENTS and CREAMS

Prepare by diluting to about 1 in 10 by emulsifying with the chosen emulsifying agent in a suitable sterile diluent such as *Fluid A (see Diluting and Rinsing Fluids for Membrane Filtration).* Transfer the diluted product to a medium not containing an emulsifying agent.

Incubate the inoculated media for not less than 14 days. Observe the cultures several times during the incubation period. Shake cultures containing oily products gently each day. However, when Fluid Thioglycollate Medium is used for the detection of anaerobic microorganisms, keep shaking or mixing to a minimum in order to maintain anaerobic conditions.

CATGUT and OTHER SURGICAL SUTURES FOR VETERINARIAN USE

Use for each medium not less than the quantities of the product prescribed in Tables 2 and 3. Open the sealed package using aseptic precautions, and remove three sections of the strand for each culture medium. Carry out the test on three sections, each 30-cm long, which have been cut off from the beginning, the center, and the end of the strand. Use whole strands from freshly opened cassette packs. Transfer each section of the strand to the selected medium. Use sufficient medium to cover adequately the material to be tested (20 mL to 150 mL).

*SOLIDS*

Transfer a quantity of the product in the form of a dry solid (or prepare a suspension of the product by adding sterile diluent to the immediate container), corresponding to not less than the quantity indicated in Tables 2 and 3. Transfer the material so obtained to 200 mL of Fluid Thioglycollate Medium, and mix. Similarly, transfer the same quantity to 200 mL of Soybean–Casein Digest Medium, and mix. Proceed as directed above.

PURIFIED COTTON, GAUZE, SURGICAL DRESSINGS, and RELATED ARTICLES

From each package of cotton, rolled gauze bandage, or large surgical dressings being tested, aseptically remove two or more portions of 100- to 500-mg each from the innermost part of the sample. From individually packaged, single-use materials, aseptically remove the entire article. Immerse the portions or article in each medium, and proceed as directed above.

STERILE DEVICES

Articles can be immersed intact or disassembled. To ensure that device pathways are also in contact with the media, immerse the appropriate number of units per medium in a volume of medium sufficient to immerse the device completely, and proceed as directed above. For extremely large devices, immerse those portions of the device that are to come into contact with the patient in a volume of medium sufficient to achieve complete immersion of those portions.

For catheters where the inside lumen and outside are required to be sterile, either cut them into pieces such that the medium is in contact with the entire lumen or fill the lumen with medium, and then immerse the intact unit.

OBSERVATION AND INTERPRETATION OF RESULTS

At intervals during the incubation period and at its conclusion, examine the media for macroscopic evidence of microbial growth. If the material being tested renders the medium turbid so that the presence or absence of microbial growth cannot be readily determined by visual examination, 14 days after the beginning of incubation transfer portions (each not less than 1 mL) of the medium to fresh vessels of the same medium, and then incubate the original and transfer vessels for not less than 4 days.
If no evidence of microbial growth is found, the product to be examined complies with the test for sterility. If evidence of microbial growth is found, the product to be examined does not comply with the test for sterility, unless it can be clearly demonstrated that the test was invalid for causes unrelated to the product to be examined. The test may be considered invalid only if one or more of the following conditions are fulfilled:

1. The data of the microbiological monitoring of the sterility testing facility show a fault.
2. A review of the testing procedure used during the test in question reveals a fault.
3. Microbial growth is found in the negative controls.
4. After determination of the identity of the microorganisms isolated from the test, the growth of this species (or these species) may be ascribed unequivocally to faults with respect to the material and or the technique used in conducting the sterility test procedure.

If the test is declared to be invalid, it is repeated with the same number of units as in the original test. If no evidence of microbial growth is found in the repeat test, the product examined complies with the test for sterility. If microbial growth is found in the repeat test, the product examined does not comply with the test for sterility.

APPLICATION OF THE TEST TO PARENTERAL PREPARATIONS, OPHTHALMIC, AND OTHER NONINJECTABLE PREPARATIONS REQUIRED TO COMPLY WITH THE TEST FOR STERILITY

When using the technique of membrane filtration, use, whenever possible, the whole contents of the container, but not less than the quantities indicated in Table 2, diluting where necessary to about 100 mL with a suitable sterile solution, such as “Fluid A” (see Diluting and Rinsing Fluids for Membrane Filtration).

When using the technique of direct inoculation of media, use the quantities shown in Table 2, unless otherwise justified and authorized. The tests for bacterial and fungal sterility are carried out on the same sample of the product to be examined. When the volume or the quantity in a single container is insufficient to carry out the tests, the contents of two or more containers are used to inoculate the different media.

MINIMUM NUMBER OF ITEMS TO BE TESTED

The minimum number of items to be tested in relation to the size of the batch is given in Table 3.

(85) BACTERIAL ENDOTOXINS TEST

*Portions of this general chapter have been harmonized with the corresponding texts of the European Pharmacopoeia and/or the Japanese Pharmacopoeia. Those portions that are not harmonized are marked with symbols (*,+) to specify this fact.

The Bacterial Endotoxins Test (BET) is a test to detect or quantify endotoxins from Gram-negative bacteria using amoebocyte lysate from the horseshoe crab (Limulus polyphemus or Tachypleus tridentatus).

There are three techniques for this test: the gel-clot technique, which is based on gel formation; the turbidimetric technique, based on the development of turbidity after cleavage of an endogenous substrate; and the chromogenic technique, based on the development of color after cleavage of a synthetic peptide-chromogen complex. Proceed by any of the three techniques for the test. In the event of doubt or dispute, the final decision is made based upon the gel-clot limit test unless otherwise indicated in the monograph for the product being tested. The test is carried out in a manner that avoids endotoxin contamination.

APPARATUS

Depyrogenate all glassware and other heat-stable materials in a hot air oven using a validated process. A commonly used minimum time and temperature is 30 min at 250°. If employing plastic apparatus, such as microplates and pipet tips for automatic pipetters, use apparatus that is shown to be free of detectable endotoxin and does not interfere in the test. [NOTE—In this chapter, the term “tube” includes any other receptacle such as a microtiter well.]

REAGENTS AND TEST SOLUTIONS

Amoebocyte Lysate

A lyophilized product obtained from the lysate of amoebocytes (white blood cells) from the horseshoe crab (Limulus polyphemus or Tachypleus tridentatus). This reagent refers only to a product manufactured in accordance with the regulations of the competent authority. [NOTE—Amoebocyte Lysate reacts to some β-glucans in addition to endotoxins. Amoebocyte Lysate preparations that do not react to glucans are available: they are prepared by removing the G factor reacting to glucans from Amoebocyte Lysate or by inhibiting the G factor reacting system of Amoebocyte Lysate and may be used for endotoxin testing in the presence of glucans.]

*1 For a validity test of the procedure for inactivating endotoxins, see Dry-Heat Sterilization under Sterility Assurance (1211). Use Lysate TS having a sensitivity of not less than 0.15 Endotoxin Unit per mL.
Water for Bacterial Endotoxins Test (BET)

Use Water for Injection or water produced by other procedures that shows no reaction with the lysate employed, at the detection limit of the reagent.

Lysate TS

Dissolve Amoebocyte Lysate in Water for BET, or in a buffer recommended by the lysate manufacturer, by gentle stirring. Store the reconstituted lysate, refrigerated or frozen, according to the specifications of the manufacturer.

PREPARATION OF SOLUTIONS

Standard Endotoxin Stock Solution

A Standard Endotoxin Stock Solution is prepared from a USP Endotoxin Reference Standard that has been calibrated to the current WHO International Standard for Endotoxin. Follow the specifications in the package leaflet and on the label for preparation and storage of the Standard Endotoxin Stock Solution. Endotoxin is expressed in Endotoxin Units (EU). [Note—One USP Endotoxin Unit (EU) is equal to one International Unit (IU) of endotoxin.]

Standard Endotoxin Solutions

After mixing the Standard Endotoxin Stock Solution vigorously, prepare appropriate serial dilutions of Standard Endotoxin Solution, using Water for BET. Use dilutions as soon as possible to avoid loss of activity by adsorption.

Sample Solutions

Prepare the Sample Solutions by dissolving or diluting drugs using Water for BET. Some substances or preparations may be more appropriately dissolved, or diluted in other aqueous solutions. If necessary, adjust the pH of the solution to be examined (or dilution thereof) so that the pH of the mixture of the lysate and Sample Solution falls within the pH range specified by the lysate manufacturer, usually 6.0–8.0. The pH may be adjusted by use of an acid, base, or suitable buffer as recommended by the lysate manufacturer. Acids and bases may be prepared from concentrates or solids with Water for BET in containers free of detectable endotoxin. Buffers must be validated to be free of detectable endotoxin and interfering factors.

DETERMINATION OF MAXIMUM VALID DILUTION (MVD)

The maximum valid dilution is the maximum allowable dilution of a specimen at which the endotoxin limit can be determined. Determine the MVD from the following equation:

$$MVD = \frac{\text{endotoxin limit} \times \text{concentration of Sample Solution}}{\lambda}$$

Endotoxin Limit

The endotoxin limit for parenteral drugs, defined on the basis of dose, equals \(K/M^{\frac{2}{3}}\), where \(K\) is a threshold pyrogenic dose of endotoxin per kg of body weight, and \(M\) is equal to the maximum recommended bolus dose of product per kg of body weight. When the product is to be injected at frequent intervals or infused continuously, \(M\) is the maximum total dose administered in a single hour period. The endotoxin limit for parenteral drugs is specified in the individual monograph in units such as EU/mL, EU/mg, EU/Unit of biological activity, etc.

Concentration of Sample Solution

- mg/mL: in the case of endotoxin limit specified by weight (EU/mg);
- Units/mL: in the case of endotoxin limit specified by unit of biological activity (EU/Unit);
- mL/mL: when the endotoxin limit is specified by volume (EU/mL).

\(\lambda\): the labeled sensitivity in the Gel-Clot Technique (EU/mL) or the lowest concentration used in the standard curve for the Turbidimetric Technique or Chromogenic Technique.

\(^{2}\) \(K\) is 5 USP-EU/kg of body weight for any route of administration other than intrathecal (for which \(K\) is 0.2 USP-EU/kg of body weight). For radiopharmaceutical products not administered intrathecally, the endotoxin limit is calculated as 175 EU/V, where \(V\) is the maximum recommended dose in mL. For intrathecally administered radiopharmaceuticals, the endotoxin limit is obtained by the formula 14 EU/V. For formulations (usually anticancer products) administered on a per square meter of body surface, the formula is \(K/M\), where \(K = 100\) EU/m² and \(M\) is the maximum dose/m².
GEL-CLOT TECHNIQUE

The gel-clot technique is used for detecting or quantifying endotoxins based on clotting of the lysate reagent in the presence of endotoxin. The minimum concentration of endotoxin required to cause the lysate to clot under standard conditions is the labeled sensitivity of the lysate reagent. To ensure both the precision and validity of the test, perform the tests for confirming the labeled lysate sensitivity and for interfering factors as described in Preparatory Testing, immediately below.

Preparatory Testing

TEST FOR CONFIRMATION OF LABELED LYSATE SENSITIVITY

Confirm in four replicates the labeled sensitivity, $\lambda$, expressed in EU/mL of the lysate prior to use in the test. The test for confirmation of lysate sensitivity is to be carried out when a new batch of lysate is used or when there is any change in the test conditions that may affect the outcome of the test. Prepare standard solutions having at least four concentrations equivalent to $2\lambda$, $\lambda$, $0.5\lambda$, and $0.25\lambda$ by diluting the USP Endotoxin RS with Water for BET.

Mix a volume of the Lysate TS with an equal volume (such as 0.1-mL aliquots) of one of the Standard Endotoxin Solutions in each test tube. When single test vials or ampuls containing lyophilized lysate are used, add solutions directly to the vial or ampul. Incubate the reaction mixture for a constant period according to the directions of the lysate manufacturer (usually at $37 \pm 1^\circ$ for $60 \pm 2$ min), avoiding vibration. To test the integrity of the gel, take each tube in turn directly from the incubator, and invert it through about $180^\circ$ in one smooth motion. If a firm gel has formed that remains in place upon inversion, record the result as positive. A result is negative if an intact gel is not formed. The test is considered valid when the lowest concentration of the standard solutions shows a negative result in all replicate tests.

The endpoint is the smallest concentration in the series of decreasing concentrations of standard endotoxin that clots the lysate. Determine the geometric mean endpoint by calculating the mean of the logarithms of the endpoint concentrations of the four replicate series and then taking the antilogarithm of the mean value, as indicated in the following formula:

$$\text{geometric mean endpoint concentration} = \text{antilog} \left( \frac{\Sigma e}{f} \right)$$

where $\Sigma e$ is the sum of the log endpoint concentrations of the dilution series used, and $f$ is the number of replicate test tubes. The geometric mean endpoint concentration is the measured sensitivity of the lysate (in EU/mL). If this is not less than $0.5\lambda$ and not more than $2\lambda$, the labeled sensitivity is confirmed and is used in tests performed with this lysate.

TEST FOR INTERFERING FACTORS

Usually prepare solutions (A–D) as shown in Table 1, and perform the inhibition/enhancement test on the Sample Solutions at a dilution less than the MVD, not containing any detectable endotoxins, operating as described for Test for Confirmation of Labeled Lysate Sensitivity. The geometric mean endpoint concentrations of Solutions B and C are determined using the formula described in the Test for Confirmation of Labeled Lysate Sensitivity. The test for interfering factors must be repeated when any condition changes that is likely to influence the result of the test.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Endotoxin Concentration/ Solution to Which Endotoxin Is Added</th>
<th>Diluent</th>
<th>Dilution Factor</th>
<th>Endotoxin Concentration</th>
<th>Number of Replicates</th>
</tr>
</thead>
<tbody>
<tr>
<td>A$^a$</td>
<td>None/Sample Solution</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>4</td>
</tr>
<tr>
<td>B$^b$</td>
<td>$2\lambda$/Sample Solution</td>
<td>Sample Solution</td>
<td>1</td>
<td>$2\lambda$</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td>$1\lambda$</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4</td>
<td>$0.5\lambda$</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>8</td>
<td>$0.25\lambda$</td>
<td>4</td>
</tr>
<tr>
<td>C$^c$</td>
<td>$2\lambda$/Water for BET</td>
<td>Water for BET</td>
<td>1</td>
<td>$2\lambda$</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td>$1\lambda$</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4</td>
<td>$0.5\lambda$</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>8</td>
<td>$0.25\lambda$</td>
<td>2</td>
</tr>
<tr>
<td>D$^d$</td>
<td>None/Water for BET</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>2</td>
</tr>
</tbody>
</table>

$^a$ Solution A: A Sample Solution of the preparation under test that is free of detectable endotoxins.

$^b$ Solution B: Test for interference.

$^c$ Solution C: Control for labeled lysate sensitivity.

$^d$ Solution D: Negative control of Water for BET.

The test is considered valid when all replicates of Solutions A and D show no reaction and the result of Solution C confirms the labeled sensitivity.
If the sensitivity of the lysate determined in the presence of Solution B is not less than 0.5\(\lambda\) and not greater than 2\(\lambda\), the Sample Solution does not contain factors that interfere under the experimental conditions used. Otherwise, the Sample Solution to be examined interferes with the test.

If the sample under test does not comply with the test at a dilution less than the MVD, repeat the test using a greater dilution, not exceeding the MVD. The use of a more sensitive lysate permits a greater dilution of the sample to be examined, and this may contribute to the elimination of interference.

Interference may be overcome by suitable treatment such as filtration, neutralization, dialysis, or heating. To establish that the chosen treatment effectively eliminates interference without loss of endotoxins, perform the assay described above using the preparation to be examined to which Standard Endotoxin has been added and which has then been submitted to the chosen treatment.

**Limit Test**

**PROCEDURE**

Prepare Solutions A, B, C, and D as shown in Table 2, and perform the test on these solutions following the procedure above for Preparatory Testing, Test for Confirmation of Labeled Lysate Sensitivity.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Endotoxin Concentration/Solution to Which Endotoxin Is Added</th>
<th>Number of Replicates</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>None/Diluted Sample Solution</td>
<td>2</td>
</tr>
<tr>
<td>B</td>
<td>2(\lambda)/Diluted Sample Solution</td>
<td>2</td>
</tr>
<tr>
<td>C</td>
<td>2(\lambda)/Water for BET</td>
<td>2</td>
</tr>
<tr>
<td>D</td>
<td>None/Water for BET</td>
<td>2</td>
</tr>
</tbody>
</table>

*Prepare Solution A and the positive product control Solution B using a dilution not greater than the MVD and treatments as described for the Test for Interfering Factors in Preparatory Testing. The positive control Solutions B and C contain the Standard Endotoxin Solution at a concentration corresponding to twice the labeled lysate sensitivity. The negative control Solution D consists of Water for BET.

**INTERPRETATION**

The test is considered valid when both replicates of Solutions B and C are positive and those of Solution D are negative. When a negative result is found for both replicates of Solution A, the preparation under test complies with the test. When a positive result is found for both replicates of Solution A, the preparation under test does not comply with the test.

When a positive result is found for one replicate of Solution A and a negative result is found for the other, repeat the test. In the repeat test, the preparation under test complies with the test if a negative result is found for both replicates of Solution A. The preparation does not comply with the test if a positive result is found for one or both replicates of Solution A. However, if the preparation does not comply with the test at a dilution less than the MVD, the test may be repeated using a greater dilution, not exceeding the MVD.

**Quantitative Test**

**PROCEDURE**

The test quantifies bacterial endotoxins in Sample Solutions by titration to an endpoint. Prepare Solutions A, B, C, and D as shown in Table 3, and test these solutions by following the procedure in Preparatory Testing, Test for Confirmation of Labeled Lysate Sensitivity.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Endotoxin Concentration/Solution to Which Endotoxin Is Added</th>
<th>Diluent</th>
<th>Dilution Factor</th>
<th>Endotoxin Concentration</th>
<th>Number of Replicates</th>
</tr>
</thead>
<tbody>
<tr>
<td>A*</td>
<td>None/Sample Solution</td>
<td>Water for BET</td>
<td>1</td>
<td>—</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td>—</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4</td>
<td>—</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>8</td>
<td>—</td>
<td>2</td>
</tr>
<tr>
<td>B*</td>
<td>2(\lambda)/Sample Solution</td>
<td>—</td>
<td>1</td>
<td>2(\lambda)</td>
<td>2</td>
</tr>
<tr>
<td>C*</td>
<td>2(\lambda)/Water for BET</td>
<td>Water for BET</td>
<td>1</td>
<td>2(\lambda)</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td>1(\lambda)</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4</td>
<td>0.5(\lambda)</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>8</td>
<td>0.25(\lambda)</td>
<td>2</td>
</tr>
</tbody>
</table>

*Not official text. Please refer to the currently official version of the applicable USP–NF or FCC standard for compliance purposes. Published on March 26, 2020.
Table 3. Preparation of Solutions for the Gel-Clot Assay (continued)

<table>
<thead>
<tr>
<th>Solution</th>
<th>Endotoxin Concentration/ Solution to Which Endotoxin Is Added</th>
<th>Diluent</th>
<th>Dilution Factor</th>
<th>Endotoxin Concentration</th>
<th>Number of Replicates</th>
</tr>
</thead>
<tbody>
<tr>
<td>D&lt;sup&gt;a&lt;/sup&gt;</td>
<td>None/Water for BET</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>2</td>
</tr>
</tbody>
</table>

<sup>a</sup> Solution A: Sample Solution under test at the dilution, not to exceed the MVD, with which the Test for Interfering Factors was completed. Subsequent dilution of the Sample Solution must not exceed the MVD. Use Water for BET to make a dilution series of four tubes containing the Sample Solution under test at concentrations of 1, ½, ¼, and ¼ relative to the concentration used in the Test for Interfering Factors. Other dilutions up to the MVD may be used as appropriate.

<sup>b</sup> Solution B: Solution A containing standard endotoxin at a concentration of 2<sub>λ</sub> (positive product control).

<sup>c</sup> Solution C: Two replicates of four tubes of Water for BET containing the standard endotoxin at concentrations of 2<sub>λ</sub>, 0.5<sub>λ</sub>, and 0.25<sub>λ</sub>, respectively.

<sup>d</sup> Solution D: Water for BET (negative control).

**CALCULATION AND INTERPRETATION**

The test is considered valid when the following three conditions are met: (1) Both replicates of negative control Solution D are negative; (2) Both replicates of positive product control Solution B are positive; and (3) The geometric mean endpoint concentration of Solution C is in the range of 0.5<sub>λ</sub> to 2<sub>λ</sub>.

To determine the endotoxin concentration of Solution A, calculate the endpoint concentration for each replicate by multiplying each endpoint dilution factor by <sub>λ</sub>. The endotoxin concentration in the Sample Solution is the endpoint concentration of the replicates. If the test is conducted with a diluted Sample Solution, calculate the concentration of endotoxin in the original Sample Solution by multiplying by the dilution factor. If none of the dilutions of the Sample Solution is positive in a valid assay, report the endotoxin concentration as less than <sub>λ</sub> (if the diluted sample was tested, report as less than <sub>λ</sub> times the lowest dilution factor of the sample). If all dilutions are positive, the endotoxin concentration is reported as equal to or greater than the greatest dilution factor multiplied by <sub>λ</sub> (e.g., initial dilution factor times eight times <sub>λ</sub> in Table 3).

The preparation under test meets the requirements of the test if the concentration of endotoxin in both replicates is less than that specified in the individual monograph.

**PHOTOMETRIC QUANTITATIVE TECHNIQUES**

**Turbidimetric Technique**

This technique is a photometric assay measuring increases in reactant turbidity. On the basis of the particular assay principle employed, this technique may be classified as either an endpoint-turbidimetric assay or a kinetic-turbidimetric assay. The endpoint-turbidimetric assay is based on the quantitative relationship between the concentration of endotoxins and the turbidity (absorbance or transmission) of the reaction mixture at the end of an incubation period. The kinetic-turbidimetric assay is a method to measure either the time (onset time) needed to reach a predetermined absorbance or transmission of the reaction mixture, or the rate of turbidity development. The test is carried out at the incubation temperature recommended by the lysate manufacturer (which is usually 37 ± 1°).

**Chromogenic Technique**

This technique is an assay to measure the chromophore released from a suitable chromogenic peptide by the reaction of endotoxins with lysate. On the basis of the particular assay principle employed, this technique may be classified as either an endpoint-chromogenic assay or a kinetic-chromogenic assay. The endpoint-chromogenic assay is based on the quantitative relationship between the concentration of endotoxins and the release of chromophore at the end of an incubation period. The kinetic-chromogenic assay is a method to measure either the time (onset time) needed to reach a predetermined absorbance or transmission of the reaction mixture, or the rate of color development. The test is carried out at the incubation temperature recommended by the lysate manufacturer (which is usually 37 ± 1°).

**Preparatory Testing**

To assure the precision or validity of the turbidimetric and chromogenic techniques, preparatory tests are conducted to verify that the criteria for the standard curve are valid and that the sample solution does not interfere with the test. Validation for the test method is required when conditions that are likely to influence the test result change.

**ASSURANCE OF CRITERIA FOR THE STANDARD CURVE**

The test must be carried out for each lot of lysate reagent. Using the Standard Endotoxin Solution, prepare at least three endotoxin concentrations within the range indicated by the lysate manufacturer to generate the standard curve. Perform the assay using at least three replicates of each standard endotoxin concentration according to the manufacturer’s instructions for the lysate (volume ratios, incubation time, temperature, pH, etc.). If the desired range is greater than two logs in the kinetic methods, additional standards should be included to bracket each log increase in the range of the standard curve. The absolute value of the correlation coefficient, <sub>r</sub>, must be greater than or equal to 0.980 for the range of endotoxin concentrations set up.
TEST FOR INTERFERING FACTORS

Select an endotoxin concentration at or near the middle of the endotoxin standard curve. Prepare Solutions A, B, C, and D as shown in Table 4. Perform the test on Solutions A, B, C, and D at least in duplicate, according to the instructions for the lysate employed, for example, concerning volume of Sample Solution and Lysate TS, volume ratio of Sample Solution to Lysate TS, incubation time, etc.

Table 4. Preparation of Solutions for the Inhibition/Enhancement Test for Photometric Techniques

<table>
<thead>
<tr>
<th>Solution</th>
<th>Endotoxin Concentration</th>
<th>Solution to Which Endotoxin Is Added</th>
<th>Number of Replicates</th>
</tr>
</thead>
<tbody>
<tr>
<td>A(^a)</td>
<td>None</td>
<td>Sample Solution</td>
<td>Not less than 2</td>
</tr>
<tr>
<td>B(^b)</td>
<td>Middle concentration of the standard curve</td>
<td>Sample Solution</td>
<td>Not less than 2</td>
</tr>
<tr>
<td>C(^c)</td>
<td>At least three concentrations (lowest concentration is designated (\lambda))</td>
<td>Water for BET</td>
<td>Each not less than 2</td>
</tr>
<tr>
<td>D(^d)</td>
<td>None</td>
<td>Water for BET</td>
<td>Not less than 2</td>
</tr>
</tbody>
</table>

\(^a\) Solution A: The Sample Solution may be diluted not to exceed MVD.
\(^b\) Solution B: The preparation under test at the same dilution as Solution A, containing added endotoxin at a concentration equal to or near the middle of the standard curve.
\(^c\) Solution C: The standard endotoxin at the concentrations used in the validation of the method described for Assurance of Criteria for the Standard Curve under Preparatory Testing (positive controls).
\(^d\) Solution D: Water for BET (negative control).

The test is considered valid when the following conditions are met.
1. The absolute value of the correlation coefficient of the standard curve generated using Solution C is greater than or equal to 0.980.
2. The result with Solution D does not exceed the limit of the blank value required in the description of the lysate reagent employed, or it is less than the endotoxin detection limit of the lysate reagent employed.

Calculate the mean recovery of the added endotoxin by subtracting the mean endotoxin concentration in the solution, if any (Solution A, Table 4), from that containing the added endotoxin (Solution B, Table 4). In order to be considered free of factors that interfere with the assay under the conditions of the test, the measured concentration of the endotoxin added to the Sample Solution must be within 50%–200% of the known added endotoxin concentration after subtraction of any endotoxin detected in the solution without added endotoxin.

When the endotoxin recovery is out of the specified range, the Sample Solution under test is considered to contain interfering factors. Then, repeat the test using a greater dilution, not exceeding the MVD. Furthermore, interference of the Sample Solution or diluted Sample Solution not to exceed the MVD may be eliminated by suitable validated treatment such as filtration, neutralization, dialysis, or heat treatment. To establish that the chosen treatment effectively eliminates interference without loss of endotoxins, perform the assay described above, using the preparation to be examined to which Standard Endotoxin has been added and which has then been submitted to the chosen treatment.

Test Procedure

Follow the procedure described for Test for Interfering Factors under Preparatory Testing, immediately above.

Calculation

Calculate the endotoxin concentration of each of the replicates of Solution A, using the standard curve generated by the positive control Solution C. The test is considered valid when the following three requirements are met.
1. The results of the control Solution C comply with the requirements for validation defined for Assurance of Criteria for the Standard Curve under Preparatory Testing.
2. The endotoxin recovery, calculated from the concentration found in Solution B after subtracting the concentration of endotoxin found in Solution A, is within the range of 50%–200%.
3. The result of the negative control Solution D does not exceed the limit of the blank value required in the description of the lysate employed, or it is less than the endotoxin detection limit of the lysate reagent employed.

Interpretation

In photometric assays, the preparation under test complies with the test if the mean endotoxin concentration of the replicates of Solution A, after correction for dilution and concentration, is less than the endotoxin limit for the product.

**USP Reference Standards** (11)
USP Endotoxin RS
Fetal bovine serum (FBS) is the light-brown liquid fraction of clotted fetal bovine blood. It is depleted of cells, fibrin, and clotting factors. Although the complete composition of FBS is undefined, FBS contains high levels of growth factors and low levels of immunoglobulins. In addition, it contains other key ingredients that are essential in supporting proliferation of cells in culture. This product is used both in life science basic research and industrial manufacturing. FBS is a by-product of the meat industry and is collected from bovine fetuses removed from cattle found to be pregnant at slaughter. FBS is harvested from abattoirs that are inspected by the competent authority in the country of origin. Trained personnel following written and approved procedures should perform collection and processing. Blood is collected in a closed system in a dedicated area within the facility, and processed quickly to prevent hemolysis. The blood is allowed to clot and then typically is centrifuged in a refrigerated centrifuge to separate the serum from the other components. Serum typically is removed from the clot, transferred to labeled containers, and frozen. All manufacturers employ sterile filtration before final packaging. Additionally, gamma irradiation provides the highest assurance of the absence of viral activity. Gamma irradiation doses of 25–40 kGy provide significant log reduction of viral and other adventitious agents while preserving cellular growth performance.

The screening of FBS for viral contamination is accomplished by using all applicable testing described in the Code of Federal Regulations 9 CFR 113.53 (known as full 9 CFR testing). Mycoplasma assays are performed as described in Mycoplasma Tests (63).

IDENTIFICATION

• IDENTIFICATION—RADIAL IMMUNODIFFUSION
  Reagents
  • FBS test samples
  • Horse serum, negative control samples
  • Bovine IgG calibrator (500 mg/L)
  • Sheep albumin diluent (1% Sheep albumin, 0.18% EDTA, 1.75% NaCl, and 1.21% Tris/HCl pH 7.4).

Materials/Apparatus: Ring measuring device is calibrated in 0.1-mm increments. Radial immunodiffusion (RID) plates are commercially available and contain anti-bovine IgG antiserum in a 1.5% agarose gel, 0.1 M phosphate buffer, pH 7.0, 0.1% sodium azide as bacteriostatic agent, and 1 μg/mL amphotericin B as an antifungal agent. Store at 2°–8°. Use RID plates that can measure bovine IgG in the range of 50–500 mg/L.

Standard curve: Use the bovine IgG calibrators for system suitability and for generation of a calibration curve. Prepare two dilutions from a 500 mg/L bovine IgG stock solution. Dilute 120 μL of the 500 mg/L stock with 80 μL of diluent (medium dilution) and 25 μL of the 500 mg/L stock with 225 μL diluent (low dilution). Label each dilution respectively as 300 mg/L and 50 mg/L calibrators. Use the 500 mg/L, 300 mg/L, and 50 mg/L solutions to generate the standard curve.

NOTE—Prepare and analyse the calibrator bovine IgG solutions in duplicate.] Load 5 μL of each sample into the 2.5-mm wells of the plate. At 72 h of incubation, measure ring diameters to the nearest 0.1 mm using an appropriate ring measuring device. Record the results and proceed to the generation of a standard curve.

The ring diameter should develop to completion at room temperature for 72 h. Using the result from each data point of the standard curve, generate a single linearity plot where y is the squared diameter (mm²) of precipitin ring around the well and x is the Bovine IgG concentration (mg/L). Calculate the linear least-squares-fit regression line of the form $y = mx + b$ with the help of suitable software and determine the values for slope (m), y-intercept (b), and coefficient of determination ($R^2$). The standard curve for the method is linear if $R^2 \geq 0.98$.

Analysis: Frozen undiluted samples of FBS are thawed and tested within 24 h if stored at 4°. Testing of FBS test and USP Fetal Bovine Serum RS samples is performed in triplicate. Prepare RID plates containing anti-bovine IgG to be tested for the various types of sera. Allow plates and reagents to equilibrate to room temperature before use by leaving the plates open for 10–15 min at room temperature to allow any condensation in the wells or on the gel surface to evaporate. Samples should not be applied to wells where moisture is visible. Prepare serial dilutions, if necessary, of FBS test and USP Fetal Bovine Serum RS samples in diluent. Dilute the negative control horse serum in diluent. Load 5 μL of each sample into the 2.5-mm wells of the plate, and incubate at room temperature for 72 h. [NOTE—The test samples and the negative control are loaded on the same plate.]

Calculation: After 72 h, measure the diameters of the rings using the ring measuring device, and record the results. Using the regression equation developed under standard curve deviation, calculate the concentration of bovine IgG in FBS samples. Concentration is expressed as mg/L.

Acceptance criteria: Horse serum is negative (should not give a precipitation ring). FBS test and USP Fetal Bovine Serum RS samples are positive and contain NMT 500 mg/L of IgG.

• HEMOGLOBIN CONTENT

(See Ultraviolet-Visible Spectroscopy (857).)

Sample preparation: FBS samples are thawed, are stored at 4°, and are tested within the same day.

Analysis: Determine the absorbance of the serum sample using a spectrophotometric cell of 1-cm path length at the wavelengths of absorbance at 576, 623, and 700 nm and using water as a blank. Calculate the concentration of hemoglobin in mg/dL:

$\text{Abs}_{576} \times 115 - \text{Abs}_{623} \times 102 - \text{Abs}_{700} \times 39.1$

Acceptance criteria: NMT 30 mg/dL
SPECIFIC TESTS

• FBS Functionality Tests

In the absence of a user-defined functionality assay, the following tests are suitable to determine the functionality of specific lots of FBS and to aid in the optimization of the growth conditions of mammalian cell cultures in the presence of FBS. For valid functionality confirmation independent of user-specific applications, tests are performed on the specified cell lines. For in-house validation of specialized cell culture applications, cell line(s) specific to those applications should be used and characterized. Use appropriate tissue culture vessels. Two tests described in this chapter are the Growth-Promotion Curve and the Clonal Assay. The decision about which type of test or the number of tests to be performed to assess suitability of a specific lot of FBS depends on the type of cell line used. For adherent cell lines, the number of colonies at the end of the culturing period represents a good assessment of the capacity of these cells, at low concentration, to grow in the presence of a specific lot of FBS. For cell lines growing in suspension cultures, the optimum growth kinetics is measured by counting viable cells after 7 days of culture.

Cell lines: Five cell lines are recommended for use:

1. HFL1 (ATCC CCL-153) normal lung, fibroblast
2. Mv1 Lu (ATCC CCL-64) mink lung, epithelial
3. HL-60 (ATCC CCL-240) peripheral blood promyeloblast, suspension
4. VERO (ATCC CCL-81) monkey kidney fibroblast
5. CHO (CCL-61) Chinese hamster ovary

The functionality tests described are to be performed on three cell lines, two of which are drawn from the five recommended cell lines and the third of which is the cell line relevant to the user’s application. Cell lines are cultured with specific media as recommended by ATCC.

Materials

- Suitable growth vessel/container
- Biological Safety Cabinet Class II, Type A
- Cell counter/hemacytometer
- Inverted microscope with digital camera accessory
- Tissue culture vessels: T25 cm²

Preparation of cells for assays: Quick-thaw a vial in a 37° water bath, and determine cell count and viability. Prepare multiple cultures from each cell line in serum-supplemented growth medium. Incubate the cultures at 37° following instructions provided by ATCC for each of the cell lines used for the test. Examine the prepared cultures under a microscope to ensure uniform, near-confluent monolayers or suspensions. Expand cells until there are enough for assay (about 1 × 10⁷ total cells; >90% viability).

Harvesting of cultures

1. Remove and discard the growth medium, and then rinse each culture with media lacking FBS.
2. For adherent cells, add 1 mL of Trypsin/EDTA for a few minutes for cells to disperse. Incubate at 37°, if necessary. Neutralize with 1 mL culture medium containing at least 10% FBS.
3. Spin down the cells in a centrifuge. Aspirate off wash media, and resuspend cells in an appropriate volume for seeding.

Seeding of cells

1. On day 0: For the three cell lines to be tested prepare multiple cultures using seeding densities that range between 2 × 10⁴ and 2 × 10⁶ viable cells/mL. (Different inocula are chosen initially to determine optimum growth conditions. Once the appropriate inoculum is chosen, that condition is used to propagate the cells.) Following are the recommended seeding densities:
   - Low seeding density: 2 × 10⁴ viable cells/mL
   - Mid seeding density: 6 × 10⁴ viable cells/mL
   - High seeding density: 2 × 10⁵ viable cells/mL
2. Prepare cultures in triplicate for at least five time points (in days or hours according to the cell line), to determine the seeding density that will yield the optimal growth conditions for each cell line used.
3. Incubate the cultures at 37° in a humidified incubator saturated with 5% CO₂.
4. For each time point of measurement (days 0, 1, 2, 3, 4, and 7), take a photograph of each culture, in triplicate, for both the FBS test material and the USP Fetal Bovine Serum RS at each of the three concentrations for each cell line, and record the percentage of confluency for each of the conditions. [NOTE—Perform this step before trypsinization and cell counting.]
5. Harvest the cells from the three different seeding density cultures for each specific time point. For adherent cultures, harvest cells as described above.
6. Perform and record total cell count and viability for each of the nine cultures for the FBS test and the USP Fetal Bovine Serum RS for each cell line using an appropriate cell counter or hemacytometer. [NOTE—The schedule for counting may have to be changed for fast-growing cell lines or large cells that would become confluent before day 7 and/or for slow-growing lines that need to be in culture 8–10 days before reaching a plateau. Some adherent cell lines will never reach confluency.]

• Growth-Promotion Curve

Measurements of cell proliferation rates often are used to determine the response of cells to exogenous stimuli. Quantitative assessment of cell growth conditions is an important factor in monitoring consistency of culture conditions. The optimal cell concentration range for subculturing, optimum inoculum, and doubling time are parameters that can be quantified and trended. Information about the growth kinetics of a culture is critical in the design of cell-based experiments. Cultures
vary significantly in their growth properties from lag phase, log phase, and stationary phase. Document the growth characteristics of the culture during the three growth stages to determine population doubling time and cell cycle time. Cells that have entered the stationary phase may demonstrate reduced growth potential and change in morphology. Cells may become polarized and may secrete more extracellular matrix, making them difficult to remove from the substratum. Cells at the end of the log phase give the highest yield and greatest reproducibility.

Reagents
- Growth media without FBS
- FBS test samples
- Growth medium + 10% FBS
- Trypsin/EDTA solution (0.25%/0.53 mM) in Hank’s Balanced Salt Solution (HBSS)

Analysis: Once the cells have reached the end of the log phase, subculture the cells for the test. Follow the procedure described in Specific Tests, FBS Functionality Tests, Seeding of cells and prepare multiple cultures for the USP Fetal Bovine Serum RS, and test FBS for different cell lines at three seeding densities for which at least one growth curve displays a lag phase, log phase, and stationary phase and for which the log phase is linear at three or more time points.

Viable cell counts are determined on days 0, 1, 2, 3, 4, and 7.

Calculation and Data analysis: Calculate the mean viable count [cells/cm$^2$ (adherent) or cells/mL (suspension)] and the mean percent viability for each data point. Plot the data on a semi-log scale graph with the viable count on the log scale on the y-axis and days (or hours) in culture on an arithmetic scale on the x-axis. Estimate the doubling time using a growth curve that is linear over three or more time points.

Acceptance criteria: The $R^2$ value of the line should be equal to or greater than 0.98 in order to support calculation of a valid doubling time. The doubling time of the test sample should be no less than 90% of the doubling time of USP Fetal Bovine Serum RS.

CLONAL ASSAY

This assay is designed to assess the optimal growth for adherent cell lines. Plating efficiency or colony formation at low cell density is a preferred method for analyzing the proliferative capacity and survival of single cells under optimal growth conditions. This is a very sensitive test and is often used for assessing the quality of serum lots. This technique reveals differences in the growth rate within the cell population and is capable of distinguishing between changes in growth rate (colony size) and cell survival (colony number). Because of the heterogeneous cell population of some cell cultures, remember that cells grow differently as isolated colonies at low densities. Consequently, few cells survive even under ideal conditions because all cell interaction is lost. Cloning is a survival assay that is also used for optimizing growth conditions (selection of medium and serum). If it can be confirmed that a single colony arose from a single cell, then cloning efficiency can be determined.

Reagents
- Growth medium + 10% FBS (test serum)—Eagle minimum essential medium (EMEM) with 2 mM L-glutamine and Earle’s BSS adjusted to contain 1.5 g/L sodium carbonate, 0.1 mM nonessential amino acids, and sodium pyruvate containing 100 U/mL penicillin and 100 g/mL streptomycin plus 10% FBS.
- Trypsin/EDTA solution (0.25%/0.53 mM) in HBSS.
- Dulbecco’s Phosphate Buffer Saline without calcium or magnesium.
- Carbol Fuchs−Methylene Blue Solution—Mix 20 g carbol fuchsin stock in 2 L methanol and stir for 10 min (1% carbol fuchsin). Mix 50 g methylene blue in 5 L methanol, and stir for 10 min (1% methylene blue). Prepare Carbol Fuchsia−Methylene Blue working solution by mixing methylene blue, methanol, and carbol fuchsin in a ratio of 3:2:1. Mix for 20 min and filter through four folds of cheesecloth in a funnel. Aliquot and store in brown glass bottles at 15° to 25°.

Sample: Multiple lots of FBS are used for this assay. For each lot of serum to be tested, add 20 mL of FBS to 180 mL of EMEM, and use the same sample for the entire test. Sterilize using 0.22-m l protein binding filter units. Store growth medium at 4° until ready to use.

Cell preparation: This test is only for adherent cultures and is performed with the adherent cell lines described under Cell Lines (HFL1 and Mv 1 Lu). One week before testing serum, expand the cell lines as described under Seeding of Cells, change the medium every 2–3 days, and subculture the cells when they are about 90% confluent. Determine the cell count and viability (viability should be >90%) before performing the assay. Harvest cells as described under Harvesting of Cultures, wash twice, and resuspend cells in basal EMEM.

Analysis: The procedure involves plating single-cell suspension at low densities (2–50 cells/cm$^2$) from which discrete colonies will form. At the end of the assay, fix, stain, and count the number of colonies as directed below.

1. For each cell line label ten 60-mm × 15-mm tissue culture dishes for each serum lot that will be tested. Label the side of the lower half of each dish, including controls.
2. Transfer 5 mL of medium containing 10% of the appropriate test serum (10 replicates). Add 400 cells per culture dish (aim for a cell concentration of about 800 cells/mL).
3. Incubate for 10–14 days at 37° in a humidified incubator saturated with 5% CO$_2$.
4. Remove the supernatant and add enough Carbol Fuchs−Methylene Blue Solution to cover each of the culture dishes for 10 min.
5. Remove the stain; rinse the culture dishes with several changes of distilled water; invert the dishes on paper towels; and allow to dry.
6. Count and record (1) the number of colonies and (2) the total surface of stained colonies (mm$^2$). Calculate means and standard deviations.

Acceptance criteria: Percent plating efficiency is expressed by counting the number of colonies in a defined area divided by the number of cells seeded multiplied by 100. Compare results between lots of FBS, and select a serum lot that is good for various types of cells and optimal for a specific cell culture application.
• PH (791): 7.00–8.00, in undiluted serum samples
• OSMOLALITY AND OSMOLARITY (785), Osmolality: 280–360 mOsmol/kg
• BACTERIAL ENDOTOXINS TEST (85): It contains not more than 10 USP Endotoxin Units/mL of serum.
• TOTAL PROTEIN CONTENT (1057): 30–45 mg/mL
• STERILITY TESTS (71): Meets the requirements

ADDITIONAL REQUIREMENTS
• PACKAGING AND STORAGE: Store in sealed containers at a temperature of −10° or below.
• LABELING: Label it to indicate that contents are Fetal Bovine Serum, and indicate lot number, expiration date, and storage conditions. Also, indicate country of origin on product labeling.
• USP REFERENCE STANDARDS (11)
  USP Endotoxin RS
  USP Fetal Bovine Serum RS

[111] DESIGN AND ANALYSIS OF BIOLOGICAL ASSAYS

INTRODUCTION

The potency of several Pharmacopeial articles must be determined by bioassays. The aim of this chapter is to present a concise account of certain essential biometrical procedures for bioassays in chapters or monographs of USP–NF, namely outlier identification, confidence intervals for relative potency measurements, and combination of independent assays. For bioassays not in USP–NF, other methods may be appropriate. See general information chapter Analysis of Biological Assays (1034) which may be a helpful, but not mandatory, guidance.

REJECTION OF OUTLYING OR ABERRANT OBSERVATIONS

A response that is questionable because of failure to comply with the procedure during the course of an assay is rejected. Other aberrant values may be discovered only after the responses have been tabulated, but can then be traced to assay irregularities that justify their omission. The arbitrary rejection or retention of an apparently aberrant response can be a serious source of bias. In general, the rejection of observations solely on the basis of their relative magnitudes, without investigation as to cause, is a procedure to be used sparingly. Should it be understood, either following an investigation into cause or based on practical assay experience, that an observation’s discordance is unlikely to arise from a reasonable expectation of response to assay treatments, then a suspected aberrant response or outlier may be tested against one of two criteria, both of which assume that the data have an approximately normal distribution (which may be satisfied only after a suitable transformation of the original responses). Alternative statistically sound approaches to outlier detection may be used. The conditions under which outlier testing will be conducted and the criterion to be used should be specified a priori in the lab’s procedures if not specified in the monograph or chapter.

Criterion 1 (Dixon’s Test)

The first criterion is based on the variation within a single group of supposedly equivalent responses, such as a group of animals given a common concentration of a sample. At a confidence level of 99%, a valid observation will be rejected once in 100 trials (when the suspected outlier can occur at only one end) or once in 50 trials (when the suspected outlier can occur at either end), provided that relatively few, if any, responses within the group are identical. Arrange the responses in order of magnitude from $y_1$ to $y_N$, where $N$ is the number of observations in the group. Compute the relative gap by using the formulas in Table 1 below.

<table>
<thead>
<tr>
<th>Sample Size ($N$)</th>
<th>Candidate Outlier is Smallest ($y_1$)</th>
<th>Candidate Outlier is Largest ($y_N$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3–7</td>
<td>$G_1 = (y_2 - y_1)/(y_N - y_1)$</td>
<td>$G_1 = (y_N - y_{N-1})/(y_N - y_1)$</td>
</tr>
<tr>
<td>8–10</td>
<td>$G_2 = (y_2 - y_1)/(y_{N-1} - y_1)$</td>
<td>$G_2 = (y_N - y_{N-1})/(y_N - y_2)$</td>
</tr>
<tr>
<td>11–13</td>
<td>$G_3 = (y_{N-1} - y_N)/(y_{N-1} - y_1)$</td>
<td>$G_3 = (y_N - y_{N-2})/(y_N - y_2)$</td>
</tr>
</tbody>
</table>

If $G_1$, $G_2$, or $G_3$ as appropriate, exceeds the critical value in Table 2, for the observed $N$, there is a statistical basis for identifying the discordant measurement as an outlier and considering its removal. For $N$ larger than 13, use Criterion 2.

In samples from a normal population, at a confidence level of 99%, gaps equal to or larger than the following values of $G_1$, $G_2$, and $G_3$ occur with a probability $P = 0.01$, when outlier measurements can occur only at one end; or with $P = 0.02$, when they may occur at either end.

<table>
<thead>
<tr>
<th>$N$</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>$G_1$</td>
<td>0.988</td>
<td>0.889</td>
<td>0.780</td>
<td>0.698</td>
<td>0.637</td>
</tr>
</tbody>
</table>

Published on March 26, 2020
Table 2. Test for Outlier Measurements (continued)

<table>
<thead>
<tr>
<th></th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>—</th>
<th>—</th>
</tr>
</thead>
<tbody>
<tr>
<td>G₂</td>
<td>0.683</td>
<td>0.635</td>
<td>0.597</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>11</th>
<th>12</th>
<th>13</th>
<th>—</th>
<th>—</th>
</tr>
</thead>
<tbody>
<tr>
<td>G₂</td>
<td>0.679</td>
<td>0.642</td>
<td>0.615</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

Criterion 2 (Grubbs, Extreme Studentized Deviate Test)

The second criterion may be used to examine for outlying values in groups of supposedly equivalent responses and may also be used in examining the set of residuals from a fitted model (linear or nonlinear) where there is constant variance. The final model (which yields the residuals for outlier detection) should include all important design variables. (For further discussion of design variables, see general information chapter Design and Development of Biological Assays (1032), which may be a helpful, but not mandatory, resource.) (Note that for application to residuals, the following is an approximation. If the statistical software provides studentized residuals, those values should be used instead of those from the following equation.) For the value, \( R \), that is furthest from the sample mean, compute the standardized deviation \( Z \):

\[
Z = \frac{(R - \bar{R})}{S}
\]

where \( \bar{R} \) and \( S \) are the mean and standard deviation, respectively, of the set of values. For residuals from a least squares fit, such as for a parallel line assay, \( \bar{R} = 0 \), and \( S \) is the square root of the residual mean square from the analysis. If \(|Z|\) is greater than \( C \) as determined below, then the value \( R \) is identified as a statistical outlier at the 1% level.

\[
C = \frac{(N-1) t_{df-1,\alpha} \sqrt{N(N-2)}}{\sqrt{N(N-2)+t_{df-1,\alpha}^2}}
\]

where \( N \) is the sample size, \( t \) is the one-sided 100\(p\) percentage point from the \( t \) distribution with \( df \) the degrees of freedom associated with \( S \):

\[
p = 1 - \frac{0.01}{2N}
\]

Alternative outlier methods are available that are intended for use on data sets that may contain multiple outliers and for detection of outliers associated with the bioassay design or model. For further discussion of outliers, see general information chapter Analytical Data—Interpretation and Treatment (1010), which may be a helpful, but not mandatory, resource.

THE CONFIDENCE INTERVAL AND LIMITS OF POTENCY

The following method (Fieller’s) is used to determine the confidence interval for an estimate of log relative potency from a parallel line assay or a slope ratio assay. Let \( M = a/b \) be the ratio for which we need a confidence interval. For the estimates, \( a \) and \( b \), we have their respective standard errors, \( SE_a \) and \( SE_b \), and a covariance between them, denoted \( Cov \). The confidence interval, \((M_{low}, M_{up})\), for the estimated log relative potency then is as follows:

\[
\left| M - \frac{SE_a}{SE_b} + \sqrt{\frac{SE_a^2}{SE_b^2} + \frac{SE_b^2}{SE_a^2}} \right| \left| 1 - \frac{(M - a/b)^2}{SE_a^2 + SE_b^2} \right|
\]

where:

\[
g = \frac{t_{df/2} SE^2}{b^2}
\]

and \( t_{df/2} \) is the upper \( a/2 \) percentage point (or the two-sided \( a \) percentage point) with the residual degrees of freedom, \( df \), from the statistical analysis and chosen confidence level, 100\(1-(1-a)\), (usually 95%). If \( g \geq 1 \), it means that the denominator, \( b \), is not statistically significantly different from 0 and the use of the ratio is not sensible for those data. The length, \( L \), of this confidence interval is \( M_{up} - M_{low} \).

For those cases in which the estimates of \( a \) and \( b \) are statistically uncorrelated (\( Cov = 0 \)), the confidence interval formula simplifies to the following:
For further discussion of confidence intervals for potency, see chapter (1034) which may be a helpful, but not mandatory, resource.

**COMBINATION OF INDEPENDENT ASSAYS**

When the monograph or chapter permits, multiple independent assays may be performed until the combined results reduce the confidence interval width to within the limits specified in the pertinent monograph or chapter. Where two or more independent assays are required, each leading to a log-potency $M$, the $M$'s are combined using one of the following two methods.

**Method 1**

Let $M_i$ denote the logarithm of the relative potency of the $i$th assay of $h$ assay results to be combined. To combine the $h$ results, the mean, standard deviation, and standard error of the $M_i$ are calculated in the usual way:

\[
\text{Mean } \bar{M} = \frac{\sum M_i}{h}
\]

\[
\text{Standard Deviation } S = \sqrt{\frac{\sum (M_i - \bar{M})^2}{h-1}}
\]

\[
\text{Standard Error } SE = S / \sqrt{h}
\]

A $100(1 - \alpha)$% confidence interval is then found as:

\[
\bar{M} \pm t_{h-1,\alpha/2} SE
\]

where $t_{h-1,\alpha/2}$ is the upper $\alpha/2$ percentage point (or the two-sided $\alpha$ percentage point) of a $t$-distribution with $h - 1$ degrees of freedom. The width, $L$, of this interval is $2t_{h-1,\alpha/2}SE$.

**Method 2**

It is assumed that the results of each of the $h$ assays have been analyzed to give $h$ values of log potency with associated confidence limits. For each assay, $i$, obtain the confidence interval for the log potency or log relative potency. Then compute value $L_i$ by subtracting the $i$th lower confidence limit from the $i$th upper confidence limit. A weight $w_i$ for each value of the log relative potency, $M_i$, is calculated as follows, where $t_i$ has the same $t$-distribution value as that used in the calculation of confidence limits in the $i$th assay and is based on $n_i$ degrees of freedom:

\[
w_i = \frac{4t_i^2}{L_i^2}
\]

The products $w_i M_i$ are formed for each assay, and their sum is divided by the total weight $(w)$ for all assays to give the weighted mean log relative potency and its standard error as follows:

\[
\text{Mean, } \bar{M} = \frac{\sum w_i M_i}{w}
\]

\[
\text{Approximate Standard Error, } SE = 1/\sqrt{w} \quad (1)
\]

where $w = \sum w_i$

Next compute an approximate chi-square:

\[
\chi^2_M = \sum_{i=1}^{h} w_i (M_i - \bar{M})^2 = \sum_{i=1}^{h} w_i M_i^2 - w\bar{M}^2
\]

If the value of the approximate $\chi^2_M$ is well under the 5% value shown in Table 3, compute the confidence interval using the mean and approximate standard error equations in (1) above; otherwise use Alternate weights as described below. Labs need to specify in their procedures how to quantify “well under”. Absent such a specification, the 20% values of Table 3 are suggested.

A $100(1 - \alpha)$% confidence interval in the log scale is then found as:
\[ M \pm L/2 \]

where

\[ L = \frac{2t_{\alpha/2}}{\sqrt{w}} \sqrt{1 + \frac{4}{w} \sum_{i=1}^{n} w_i (w_i - w_j)} \]

\[ n' = n - 4 \frac{n - 2}{n - 1} \]

and

\[ df = \frac{w'^2}{\sum_{i=1}^{n} w_i'^2 / n_i} \]

where \( t_{\alpha/2} \) is the upper \( \alpha/2 \) percentage point (or the two-sided \( \alpha \) percentage point) of a \( t \)-distribution with degrees of freedom, \( df \). The width of this interval is \( L \).

### Table 3. Critical Values for Approximate Chi-Square Test

<table>
<thead>
<tr>
<th>( h )</th>
<th>5%</th>
<th>20%</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>3.841</td>
<td>1.642</td>
</tr>
<tr>
<td>3</td>
<td>5.991</td>
<td>3.219</td>
</tr>
<tr>
<td>4</td>
<td>7.815</td>
<td>4.642</td>
</tr>
<tr>
<td>5</td>
<td>9.488</td>
<td>5.989</td>
</tr>
<tr>
<td>6</td>
<td>11.070</td>
<td>7.289</td>
</tr>
<tr>
<td>7</td>
<td>12.592</td>
<td>8.558</td>
</tr>
<tr>
<td>8</td>
<td>14.067</td>
<td>9.803</td>
</tr>
<tr>
<td>9</td>
<td>15.507</td>
<td>11.030</td>
</tr>
<tr>
<td>10</td>
<td>16.919</td>
<td>12.242</td>
</tr>
</tbody>
</table>

**Alternate weights:** The observed variation among the estimated log potencies or relative potencies can be divided into two components:

- intra-assay variation for assay \( i \):
  \[ V_i = 1/w_i \]
- inter-assay component of variation:

\[ s_B^2 = \max \{0, \frac{1}{h - 1} \sum_{i=1}^{h} (M_i - \bar{M})^2 - \frac{1}{h} \sum_{i=1}^{h} V_i \} \]

\[ s_B^2 = \max \{0, \frac{1}{h - 1} \sum_{i=1}^{h} (M_i - \bar{M})^2 - \frac{1}{h} \sum_{i=1}^{h} V_i \} \]

For each assay, a weighting coefficient is then calculated as:

\[ w_i' = \frac{1}{V_i + s_B^2} \]

The confidence interval is then found as:

\[ \bar{M} \pm t \times SE' \]

where \( SE' = 1/\sqrt{w'} \) and \( w' = \sum_{i=1}^{n} w_i' \)

and \( t \), the \( t \)-distribution value, is often approximated by the value 2.

For further discussion of combination of assays, see (1034), which may be a helpful, but not mandatory, resource.

**APPENDIX—KEY LITERATURE**


Böhrer A. One-sided and two-sided critical values for Dixon’s outlier test for sample sizes up to \( n = 30 \). *Economic Quality Control* 23, 5–13, 2008.


〈151〉 PYROGEN TEST

INTRODUCTION

The pyrogen test is designed to limit to an acceptable level the risks of febrile reaction in the patient to the administration, by injection, of the product concerned. The test involves measuring the rise in temperature of rabbits following the intravenous injection of a test solution and is designed for products that can be tolerated by the test rabbit in a dose not to exceed 10 mL/kg injected intravenously within a period of NMT 10 min. For products that require preliminary preparation or are subject to special conditions of administration, follow the additional directions given in the individual monograph or, in the case of antibiotics or biologics, the additional directions given in the federal regulations (see Biologics (1041)). A validated, equivalent in vitro pyrogen or bacterial endotoxin test may be used in place of the in vivo rabbit pyrogen test, where appropriate.

APPARATUS AND DILUENTS

Render the syringes, needles, and glassware free from pyrogens by heating at 250° for NLT 30 min or by any other suitable method. Treat all diluents and solutions for washing and rinsing of devices or parenteral injection assemblies in a manner that will assure that they are sterile and pyrogen-free. Periodically perform control pyrogen tests on representative portions of the diluents and solutions for washing or rinsing of the apparatus. Where Sodium Chloride Injection is specified as a diluent, use Injection containing 0.9% of sodium chloride (NaCl).

TEMPERATURE RECORDING

Use an accurate temperature-sensing device such as a clinical thermometer, or thermistor probes or similar probes that have been calibrated to assure an accuracy of ±0.1° and have been tested to determine that a maximum reading is reached in less than 5 min. Insert the temperature-sensing probe into the rectum of the test rabbit to a depth of NLT 7.5 cm, and, after a period of time NLT that previously determined as sufficient, record the rabbit’s body temperature.

TEST ANIMALS

Use healthy, mature rabbits. House the rabbits individually in an area of uniform temperature between 20° and 23° and free from disturbances likely to excite them. The temperature varies NMT ±3° from the selected temperature. Before using a rabbit for the first time in a pyrogen test, condition it NMT 7 days before use by a sham test that includes all of the steps as directed in Procedure except injection. Do not use a rabbit for pyrogen testing more frequently than once every 48 h, nor prior to 2 weeks following a maximum rise of its temperature of 0.6° or more while being subjected to the pyrogen test, or following its having been given a test specimen that was adjudged pyrogenic.

PROCEDURE

Perform the test in a separate area designated solely for pyrogen testing and under environmental conditions similar to those under which the animals are housed and free from disturbances likely to excite them. Withhold all food from the rabbits used during the period of the test. Access to water is allowed at all times, but may be restricted during the test. If rectal temperature-measuring probes remain inserted throughout the testing period, restrain the rabbits with light-fitting neck stocks that allow the rabbits to assume a natural resting posture. NMT 30 min prior to the injection of the test dose, determine the “control temperature” of each rabbit: this is the base for the determination of any temperature increase resulting from the injection of a test solution. In any one group of test rabbits, use only those rabbits whose control temperatures do not vary by more than 1° from each other, and do not use any rabbit with a temperature exceeding 39.8°.

Unless otherwise specified in the individual monograph, inject into an ear vein of each of three rabbits 10 mL of the test solution per kg of body weight, completing each injection within 10 min after start of administration. The test solution is either the product, constituted if necessary as directed in the labeling, or the material under test treated as directed in the individual monograph and injected in the dose specified therein. For pyrogen testing of devices or injection assemblies, use washings or rinsings of the surfaces that come in contact with the parenterally administered material or with the injection site or internal tissues of the patient. Assure that all test solutions are protected from contamination. Perform the injection after warming the test solution to a temperature of 37 ± 2°. Record the temperature at 30-min intervals between 1 and 3 h subsequent to the injection.

TEST INTERPRETATION AND CONTINUATION

Consider any temperature decreases as zero rise. If no rabbit shows an individual rise in temperature of 0.5° or more above its respective control temperature, the product meets the requirements for the absence of pyrogens. If any rabbit shows an individual temperature rise of 0.5° or more, continue the test using five other rabbits. If NMT three of the eight rabbits show

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individual rises in temperature of 0.5° or more and if the sum of the eight individual maximum temperature rises does not exceed 3.3°, the material under examination meets the requirements for the absence of pyrogens.

RADIOACTIVE PHARMACEUTICALS

Test Dose for Preformulated, Ready-to-Use Products Labeled with Radioactivity

AGGREGATED ALBUMIN AND OTHER PARTICLE-CONTAINING PRODUCTS

For the rabbit pyrogen test, dilute the product with Sodium Chloride Injection to NLT 100 µCi/mL, and inject a dose of 3 mL/kg of body weight into each rabbit.

OTHER PRODUCTS

Where physical half-life of radionuclide is greater than 1 day: Calculate the maximum volume of the product that might be injected into a human subject. This calculation takes into account the maximum recommended radioactive dose of the product, in µCi, and the radioactive assay, in µCi/mL, of the product at its expiration date or time. Using this information, calculate the maximum volume dose per kg to a 70-kg human subject.

For the rabbit pyrogen test, inject a minimum of 10 times this dose per kg of body weight into each rabbit. If necessary, dilute with Sodium Chloride Injection. The total injected volume per rabbit is NLT 1 mL and NMT 10 mL of solution.

Where physical half-life of radionuclide is less than 1 day: For products labeled with radionuclides with a half-life of less than 1 day, the dosage calculations are identical to those described in the first paragraph in Other Products. These products may be released for distribution prior to completion of the rabbit pyrogen test, but such test must be initiated at NMT 36 h after release.

Test Dose for Pharmaceutical Constituents or Reagents to Be Labeled

The following test dose requirements pertain to reagents that are to be labeled or constituted prior to use by the direct addition of radioactive solutions such as Sodium Pertechnetate Tc 99m Injection, i.e., “cold kits”.

Assume that the entire contents of the vial of nonradioactive reagent will be injected into a 70-kg human subject, or that \( \frac{1}{70} \) of the total contents per kg will be injected. If the contents are dry, constitute with a measured volume of Sodium Chloride Injection.

For the rabbit pyrogen test, inject \( \frac{1}{7} \) of the vial contents per kg of body weight into each rabbit. The maximum dose per rabbit is the entire contents of a single vial. The total injected volume per rabbit is NLT 1 mL and NMT 10 mL of solution.

〈181〉 IDENTIFICATION—ORGANIC NITROGENOUS BASES

INTRODUCTION

The purpose of this test is the identification of tertiary amine compounds. This spectroscopic test has a limited degree of specificity and, therefore, the conformance with all additional identification tests listed in a particular monograph is necessary to ensure the identity of the specimen under examination.

ASSAY

• PROCEDURE

Standard solution: In a separator dissolve 50 mg of the corresponding USP Reference Standard in 25 mL of 0.01 N hydrochloric acid.

Sample solution: Depending upon the nature of the sample, dissolve 50 mg of the bulk substance under test in 25 mL of 0.01 N hydrochloric acid, or shake a quantity of powdered tablets or the contents of capsules, equivalent to 50 mg of the substance, with 25 mL of 0.01 N hydrochloric acid for 10 min. Transfer the liquid to a separator, filtering if necessary, and washing the filter and the residue with several small portions of water.

Instrumental conditions

(See Mid-Infrared Spectroscopy (854).)

Mode: IR

Wavelength range: 7–15 µm (1430 cm\(^{-1}\) to 650 cm\(^{-1}\))

Cell: 1-mm

Blank: Carbon disulfide

Analysis

Samples: Standard solution and Sample solution

Treat each solution as follows: Add 2 mL of 1 N sodium hydroxide and 4 mL of carbon disulfide, and shake for 2 min. Centrifuge if necessary to clarify the lower phase, and pass it through a dry filter, collecting the filtrate in a small flask provided with a glass stopper. Determine the absorption spectra of the filtered Standard solution and Sample solution without delay.

Acceptance criteria: The spectrum of the Sample solution must show all of the significant absorption bands present in the spectrum of the Standard solution.
CHEMICAL IDENTIFICATION TESTS

INTRODUCTION

Procedures in this chapter are referenced in monographs for the identification of official articles and their components. Any acids, bases, or other reagents used in these procedures should not interfere with the results. Volumes may be scaled proportionally unless otherwise indicated. All of the tests include approximate amounts, except where specified.

Instrumental techniques described in this chapter may be used in lieu of chemical identification tests. Those instrumental techniques are not exhaustive and other techniques, such as nuclear magnetic resonance, ion-selective electrodes, and near-infrared, may be used in lieu of a chemical identification test provided that they are suitable and validated.

Unless otherwise specified in the monograph, if a chemical identification test is selected for an ion, then all chemical test procedures listed for the ion shall be met. If an instrumental identification test is selected, then only one instrumental technique is required for the ion(s).

CHEMICAL IDENTIFICATION TESTS

Acetate

A. Dissolve about 30 mg of the substance to be examined in 3 mL of water, or use 3 mL of the prescribed solution. Adjust the pH of the solution with sodium hydroxide to be slightly alkaline. Add 0.25 mL of lanthanum nitrate test solution (TS). If a white precipitate is formed, filter the solution. Add successively 0.1 mL of iodine and potassium iodide TS 3, and 0.1 mL of ammonia TS 2 to the solution. If no blue color is observed, heat carefully to boiling. In the presence of acetates, a dark color develops or a blue precipitate is formed.

B. With neutral solutions of acetates, ferric chloride TS produces a red color that is destroyed by the addition of mineral acids.

Aluminum

A. By using 6 N ammonium hydroxide, solutions of aluminum salts yield a gelatinous, white precipitate that is insoluble in an excess of 6 N ammonium hydroxide.

B. 1 N sodium hydroxide or sodium sulfide TS with solutions of aluminum salts produces a similar gelatinous, white precipitate, which dissolves in an excess of either of the same reagents.

Ammonium

A. Add 0.2 g of magnesium oxide to the solution under test. Pass a current of air through the mixture, and direct the gas that escapes to just beneath the surface of the indicator solution prepared previously by mixing 1 mL of 0.1 M hydrochloric acid and 0.05 mL of methyl red TS 2. In the presence of ammonium, the color of the indicator solution is changed to yellow. After directing the gas into the indicator solution for a sufficient period of time, add 1 mL of freshly prepared sodium cobaltinitrite TS to the indicator solution. Upon the addition of the sodium cobaltinitrite TS, a yellow precipitate is formed when ammonium is present.

Antimony

A. With hydrogen sulfide, solutions of antimony(III) compounds, strongly acidified with hydrochloric acid, yield an orange precipitate of antimony sulfide that is insoluble in 6 N ammonium hydroxide but is soluble in ammonium sulfide TS.

Barium

A. Solutions of barium salts yield a white precipitate with the addition of 2 N sulfuric acid. This precipitate is insoluble in hydrochloric acid and in nitric acid.

Benozoate

A. In neutral solutions, benzoates yield a salmon-colored precipitate with ferric chloride TS.

B. In moderately concentrated solutions, benzoates yield a precipitate of benzoic acid upon acidification when 2 N sulfuric acid is added. This precipitate is readily soluble in ethyl ether.

Bicarbonate

A. Bicarbonates effervesc with acids, evolving a colorless gas that, when passed into calcium hydroxide TS, produces a white precipitate immediately.

B. A cold solution (1:20) of a soluble bicarbonate or the solution prescribed in the specific monograph remains unchanged or is only slightly colored by phenolphthalein TS.

Bismuth

A. When dissolved in a slight excess of nitric acid or hydrochloric acid, bismuth salts yield a white precipitate upon dilution with water. This precipitate is colored brown by hydrogen sulfide, and the resulting compound dissolves in a warm mixture of equal parts of nitric acid and water.

Bisulfite See Sulfite.

Borate

A. To 1 mL of a borate solution acidified with hydrochloric acid, add 3 or 4 drops of iodine TS and 3 or 4 drops of polyvinyl alcohol solution (1:50): an intense blue color is produced.

Bromide

A. Solutions of bromides, upon the addition of chlorine TS dropwise, liberate bromine, which is dissolved by shaking with chloroform, coloring the chloroform red-to-reddish brown.

B. Silver nitrate TS produces, in solutions of bromides, a yellowish-white precipitate that is insoluble in nitric acid and is slightly soluble when 6 N ammonium hydroxide is added.

Calcium

A. Solutions of calcium salts form insoluble oxalates when treated as follows. To a solution of the calcium salt (1:20) or the solution prescribed in the specific monograph, add 2 drops of methyl red TS and neutralize with 6 N ammonium hydroxide. Add 3 N hydrochloric acid dropwise, until the solution is acid to the indicator. Upon the addition of ammonium oxalate TS, a white precipitate is formed. This precipitate is insoluble when 6 N acetic acid is added but dissolves in hydrochloric acid.
Carbonate
- A. Carbonates effervesce with acids, evolving a colorless gas that, when passed into calcium hydroxide TS, produces a white precipitate immediately.
- B. A cold solution (1:20) of a soluble carbonate or the solution prescribed in the specific monograph is colored red by phenolphthalein TS, whereas a similar solution of a bicarbonate remains unchanged or is only slightly colored.

Chlorate
- A. Solutions of chlorates yield no precipitate with silver nitrate TS. The addition of sulfuric acid to this mixture produces a white precipitate that is insoluble in nitric acid but is soluble in 6 N ammonium hydroxide.
- B. Upon ignition, chlorates yield chlorides, recognizable by appropriate tests.
- C. When sulfuric acid is added to a dry chlorate, decrepitation occurs and a greenish-yellow gas is evolved. [Caution—Use only a small amount of chlorate for this test, and exercise extreme caution in performing it.]

Chloride
- A. With silver nitrate TS, solutions of chlorides yield a white, curdy precipitate that is insoluble in nitric acid but is soluble in a slight excess of 6 N ammonium hydroxide.
- B. When testing amine (including alkaloidal) hydrochlorides that do not respond to the above test, add 1 drop of diluted nitric acid and 0.5 mL of silver nitrate TS to a solution of the substance being examined containing, unless otherwise directed in the monograph, about 2 mg of chloride ion in 2 mL: a white, curdy precipitate is formed. Centrifuge the mixture without delay, and decant the supernatant layer. Wash the precipitate with three 1-mL portions of nitric acid solution (1:100), and discard the washings. Add ammonia TS dropwise to this precipitate. It dissolves readily.
- C. When a monograph specifies that an article responds to the test for dry chlorides, mix the solid to be tested with an equal weight of manganese dioxide, moisten with sulfuric acid, and gently heat the mixture: chlorine, which is recognizable by the production of a blue color with moistened starch iodide paper, is evolved.

Citrate
- A. To 15 mL of pyridine add a solution or suspension of a few mg of a citrate salt in 1 mL of water or the solution prescribed in the specific monograph, and shake. To this mixture add 5 mL of acetic anhydride, and shake: a light red color is produced.

Cobalt
- A. Solutions of cobalt salts (1:20) in 3 N hydrochloric acid or the solution prescribed in the specific monograph yield a red precipitate when heated on a steam bath with an equal volume of a hot, freshly prepared solution of 1-nitroso-2-naphthol (1:10) in 9 N acetic acid.
- B. Solutions of cobalt salts, when saturated with potassium chloride and treated with potassium nitrite and acetic acid, yield a yellow precipitate.

Copper
- A. Cupric compounds, acidified with hydrochloric acid, deposit a red film of metallic copper upon a bright, un tarnished surface of metallic iron.
- B. An excess of 6 N ammonium hydroxide, when added to a solution of a cupric salt, produces first a bluish precipitate and then a deep blue-colored solution.
- C. With potassium ferrocyanide TS, solutions of cupric salts yield a reddish-brown precipitate, insoluble in dilute acids.

Hypophosphite
- A. Hypophosphites in solution yield a white precipitate with mercuric chloride TS. This precipitate becomes gray when an excess of hypophosphite is present.
- B. Solutions of hypophosphites, acidified with sulfuric acid and warmed with cupric sulfate TS, yield a red precipitate.

Iodide
- A. Solutions of iodides, upon the addition of chlorine TS dropwise, liberate iodine, which colors the solution from yellow to red. When the solution is shaken with chloroform, the solution is colored violet. The iodine thus liberated gives a blue color with starch TS.
- B. Silver nitrate TS produces, in solutions of iodides, a yellow, curdy precipitate that is insoluble in nitric acid and insoluble in 6 N ammonium hydroxide.

Iron
- A. Ferrous and ferric compounds in solution yield a black precipitate with ammonium sulfide TS. This precipitate dissolves in cold, 3 N hydrochloric acid and evolves hydrogen sulfide.

Ferric salts
- A. Acid solutions of ferric salts yield a dark blue precipitate with potassium ferrocyanide TS.
- B. With an excess of 1 N sodium hydroxide added to the ferric salts solutions, a reddish-brown precipitate is formed.
- C. With ammonium thiocyanate TS, solutions of ferric salts produce a deep red color that is not destroyed by dilute mineral acids.

Ferrous salts
- A. Solutions of ferrous salts yield a dark blue precipitate with potassium ferricyanide TS. This precipitate is insoluble in 3 N hydrochloric acid but is decomposed by 1 N sodium hydroxide.
- B. With 1 N sodium hydroxide, solutions of ferrous salts yield a greenish-white precipitate; when shaken, the color changes rapidly to green and then to brown.

Lactate
- A. When solutions of lactates are acidified with sulfuric acid, potassium permanganate TS is added; the mixture is heated, and acetaldehyde evolves. Acetaldehyde can be detected by allowing the vapor to come into contact with a filter paper that has been moistened with a freshly prepared mixture of equal volumes of 20% aqueous morpholine and sodium nitroferricyanide TS: a blue color is produced.

Lead
- A. With 2 N sulfuric acid, solutions of lead salts yield a white precipitate that is insoluble in 3 N hydrochloric or 2 N nitric acid but is soluble in warm 1 N sodium hydroxide and in ammonium acetate TS.
• B. With potassium chromate TS, solutions of lead salts, free or nearly free from mineral acids, yield a yellow precipitate that is insoluble in 6 N acetic acid but is soluble in 1 N sodium hydroxide.

**Lithium**

- A. With sodium carbonate TS, moderately concentrated solutions of lithium salts, made alkaline with sodium hydroxide, yield a white precipitate on boiling. The precipitate is soluble in ammonium chloride TS.
- B. Solutions of lithium salts are not precipitated by 2 N sulfuric acid or soluble sulfates (distinction from strontium).

**Magnesium**

- A. Solutions of magnesium salts in the presence of ammonium chloride yield not more than a slightly hazy precipitate when neutralized with ammonium carbonate TS, but on the subsequent addition of dibasic sodium phosphate TS, a white, crystalline precipitate, which is insoluble in 6 N ammonium hydroxide, is formed.

**Manganese**

- A. With ammonium sulfide TS, solutions of manganous salts yield a salmon-colored precipitate that dissolves in acetic acid.
- B. With dilute mineral acids or with 6 N acetic acid, nitrites evolve brownish-red fumes. The solution colors upon rubbing, becomes bright and silvery in appearance.
- B. With hydrogen sulfide, solutions of mercury compounds yield a black precipitate that is insoluble in ammonium sulfide TS and in boiling 2 N nitric acid.

**Mercury**

- A. When applied to bright copper foil, solutions of mercury salts, free from an excess of nitric acid, yield a deposit that, upon standing, becomes bright and silvery in appearance.
- B. With hydrogen sulfide, solutions of mercury compounds yield a black precipitate that is insoluble in ammonium sulfide TS and in boiling 2 N nitric acid.

**Mercuric salts**

- A. Solutions of mercuric salts yield a yellow precipitate with 1 N sodium hydroxide.
- B. Solutions of mercuric salts yield, in neutral solutions with potassium iodide TS, a scarlet precipitate that is very soluble in an excess of the reagent.

**Mercurous salts**

- A. Mercurous compounds are decomposed by 1 N sodium hydroxide, producing a black color.
- B. With hydrochloric acid, solutions of mercurous salts yield a white precipitate that is blackened by the addition of 6 N ammonium hydroxide.
- C. With potassium iodide TS, a yellow precipitate, which may become green upon standing, is formed.

**Nitrate**

- A. When a solution of a nitrate is mixed with an equal volume of sulfuric acid, the mixture is cooled, and a solution of ferrous sulfate is superimposed; a brown color is produced at the junction of the two liquids.
- B. When a nitrate is heated with sulfuric acid and metallic copper, brownish-red fumes evolve.
- C. Nitrates do not decolorize acidified potassium permanganate TS (distinction from nitrites).

**Nitrite**

- A. When treated with dilute mineral acids or with 6 N acetic acid, nitrites evolve brownish-red fumes. The solution colors starch–iodide paper blue.

**Oxalate**

- A. Neutral and alkaline solutions of oxalates yield a white precipitate with calcium chloride TS. This precipitate is insoluble in 6 N acetic acid but is dissolved by hydrochloric acid.
- B. Hot acidified solutions of oxalates decolorize potassium permanganate TS.

**Permanganate**

- A. Solutions of permanganates acidified with sulfuric acid are decolorized by hydrogen peroxide TS and by sodium bisulfite TS, in the cold, and by oxalic acid TS in a hot solution.

**Peroxide**

- A. Solutions of peroxides slightly acidified with sulfuric acid yield a deep blue color upon the addition of potassium dichromate TS. On shaking the mixture with an equal volume of ethyl ether and allowing the liquids to separate, the blue color is found in the ethyl ether layer.

**Phosphate**

[Note—Where the monograph specifies the identification test for Phosphate, use the tests for orthophosphates, unless the instructions specify the use of the pyrophosphate tests or indicate that the product is to be ignited before performing the test.]

**Orthophosphates**

- A. With silver nitrate TS, neutral solutions of orthophosphates yield a yellow precipitate that is soluble in 2 N nitric acid and in 6 N ammonium hydroxide.
- B. With ammonium molybdate TS, acidified solutions of orthophosphates yield a yellow precipitate that is soluble in 6 N ammonium hydroxide. This precipitate may be slow to form.

**Pyrophosphates**

- A. With silver nitrate TS, pyrophosphates obtained by ignition yield a white precipitate that is soluble in 2 N nitric acid and soluble in 6 N ammonium hydroxide.
- B. With ammonium molybdate TS, pyrophosphates obtained by ignition yield a yellow precipitate that is soluble in 6 N ammonium hydroxide.

**Potassium**

- A. In neutral, concentrated, or moderately concentrated solutions of potassium salts (depending upon the solubility and the potassium content), sodium bitartrate TS produces a white crystalline precipitate that is soluble in 6 N ammonium hydroxide and in solutions of alkali hydroxides and carbonates. The formation of the precipitate, which is usually slow, is accelerated by stirring or rubbing the inside of the test tube with a glass rod. The addition of a small amount of glacial acetic acid or alcohol also promotes the precipitation.

**Salicylate**

- A. In moderately concentrated solutions of salicylates, ferric chloride TS produces a violet color.
- B. Addition of acids to moderately concentrated solutions of salicylates produces a white, crystalline precipitate of salicylic acid that melts between 158° and 161°.
Silver
- A. With hydrochloric acid, solutions of silver salts yield a white, curdy precipitate that is insoluble in nitric acid but is readily soluble in 6 N ammonium hydroxide.
- B. A solution of a silver salt, to which 6 N ammonium hydroxide and a small quantity of formaldehyde TS are added, deposits, upon warming, a mirror of metallic silver upon the sides of the container.

Sodium
- A. Unless otherwise specified in an individual monograph, prepare a solution to contain 0.1 g of the sodium compound in 2 mL of water. Add 2 mL of 15% potassium carbonate, and heat to boiling. No precipitate is formed. Add 4 mL of potassium pyroantimonate TS, and heat to boiling. Allow to cool in ice water and, if necessary, rub the inside of the test tube with a glass rod. A dense precipitate is formed.

Sulfate
- A. With barium chloride TS, solutions of sulfates yield a white precipitate that is insoluble in hydrochloric acid and in nitric acid.
- B. With lead acetate TS, neutral solutions of sulfates yield a white precipitate that is soluble in ammonium acetate TS.
- C. Hydrochloric acid produces no precipitate when added to solutions of sulfates (distinction from thiosulfates).

Sulfite
- A. When treated with 3 N hydrochloric acid, sulfites and bisulfites yield sulfur dioxide, which blackens filter paper moistened with mercurous nitrate TS.

Tartrate
- A. Dissolve a few mg of a tartrate salt in 2 drops of sodium metaperiodate solution (1:20). Add 1 drop of 1 N sulfuric acid and after 5 min add a few drops of sulfurous acid, followed by a few drops of fuchsin-sulfurous acid TS: a reddish-pink color is produced within 15 min.

Thiocyanate
- A. With ferric chloride TS, solutions of thiocyanates yield a red color that is not destroyed by moderately concentrated mineral acids.

Thiosulfate
- A. With hydrochloric acid, solutions of thiosulfates yield a white precipitate that soon turns yellow, and evolve sulfur dioxide, which blackens filter paper moistened with mercurous nitrate TS.
- B. The addition of ferric chloride TS to solutions of thiosulfates produces a dark violet color that disappears quickly.

Zinc
- A. In the presence of sodium acetate, solutions of zinc salts yield a white precipitate with hydrogen sulfide. This precipitate is insoluble in acetic acid but is dissolved by 3 N hydrochloric acid.
- B. Ammonium sulfide TS produces a similar precipitate in neutral and in alkaline solutions.
- C. With potassium ferrocyanide TS, zinc salts in solution yield a white precipitate that is insoluble in 3 N hydrochloric acid.

**Change to read:**

**INSTRUMENTAL IDENTIFICATION TESTS**

Instrumental techniques described in this section may be used in lieu of procedures described in Chemical Identification Tests. Instrumental techniques provide flexibility in the choice of identification tests. All instrumental techniques shall follow method validation procedures for identification tests (see Validation of Compendial Procedures (1225)), Validation, Data Elements Required for Validation, Category IV. Instrumental Identification Tests must demonstrate specificity. In addition, other suitable, validated instrumental techniques may be used.

The selection of the appropriate sample preparation depends on the material under test and must be appropriate for the technique being used. The analyst may use any of the following preparation procedures, with the appropriate verification. An electronic library spectrum of the Reference Standard may be used in comparison to the test sample provided adequate specificity is maintained. When using solvents, the solvent must be free of interfering species. Use USP Reference Standards where available (see General Notices, 5.80 USP Reference Standards).

**IDENTIFICATION USING X-RAY FLUORESCENCE SPECTROMETRY**

X-ray fluorescence spectrometry (XRF) can typically be used for identification of elements with atomic numbers from magnesium through uranium. The actual range of elements for which the instrument is capable is dependent on the instrument design. Guidance regarding the use of XRF may be found in X-Ray Fluorescence Spectrometry (735).

**Sample preparation**
- **Powders/solids:** Powders and solids can be used without further manipulation, or material can be made into pellets as described in (735).
- **Neat liquids:** Liquids can be used without further manipulation, provided the liquid is a single phase, is compatible with the sample holder, and has sufficiently low volatility.

**Samples in solution:** Samples may be dissolved in an appropriate solvent.

**Procedure**
- **Blank:** For Powders/solids and Neat liquids, prepare a blank sample holder. For Samples in solution, use the solvent as a blank.
- **Reference standard and Sample:** Prepare each Reference standard and Sample by using sufficient material as required for the specific manufacturer’s instrumentation.

**Analysis:** Analyze the Blank, Reference standard, and Sample according to the manufacturer’s suggestions for the specific instrument. If there is any interference, use the Blank to make the correction. The spectrum of the Reference standard exhibits radiation characteristic of the element(s) under investigation. The energy bands of the Sample exhibit radiation characteristic of the element(s) under investigation and compare qualitatively with that of the Reference standard.
• IDENTIFICATION USING ATOMIC SPECTROSCOPY TECHNIQUES: ATOMIC ABSORPTION SPECTROSCOPY, INDUCTIVELY COUPLED PLASMA–OPTICAL EMISSION SPECTROSCOPY, INDUCTIVELY COUPLED PLASMA–MASS SPECTROMETRY

Atomic absorption (AA) spectroscopy techniques can be used for the identification of many elements. Guidance regarding the use of AA spectroscopy may be found in Atomic Absorption Spectroscopy (852). Guidance regarding the use of inductively coupled plasma–optical emission spectroscopy (ICP–OES) or inductively coupled plasma–mass spectrometry (ICP–MS) may be found in Plasma Spectrochemistry (730).

Sample preparation: Samples should be dissolved in an appropriate solvent. If dissolving the sample is not possible, digestion may be necessary.

Procedure
Blank: Prepare an appropriate solution that does not contain the analyte(s) of interest and is compatible with the technique being used.
Standard solution: Standards must contain the analyte of interest. All solutions should be matrix-matched where possible.
Sample solution: Prepare the sample in the same solvent as the standard.

Analysis: Analyze the Blank, Standard solution, and Sample solution according to the manufacturer’s suggestions for the specific instrument. When a wavelength-based technique is used, wherever possible select at least two wavelengths characteristic of the analyte(s) of interest. When a mass-based technique is used, wherever possible select at least two isotopes characteristic of the analyte(s) of interest. If two isotopes are unavailable, or if an element is monoisotopic, it may be possible to monitor the oxide of the analyte(s) (m + 16), if one is formed. If no oxide is formed, or if multiple isotopes cannot be evaluated because of interferences or because of a limited number of isotopes, then a different technique may be required. If interfering elements are present in the sample solution, wavelengths or masses examined must be selected to unequivocally identify the analyte(s) of interest.

• IDENTIFICATION USING ION CHROMATOGRAPHY

Ion chromatography (IC) can be used to identify a number of anions and cations found in drug substances (for additional information, see Ion Chromatography (1065)).

Apparatus: Analyte detection will typically utilize suppressed conductivity, although other detection methods are possible, depending on the analyte (see (1065)). The ion-exchange column must be capable of separating the analyte from any other ions of the same charge known to be in the sample at a concentration ≥5% of the analyte.

Procedure
Blank: Use the sample solvent as a blank.
Standard and Sample solutions: Dissolve or dilute the sample in water. Other solvents can be used if compatible with the IC column.

Analysis: Analyze an equal volume of the Blank, Reference Standard, and sample (according to the manufacturer’s suggestions for the specific instrument and column dimensions). The counterion is identified if a peak in the sample has the same retention time as the peak in the Reference Standard, and there is not a peak of ≥5% of its size at the same retention time in the Blank.

• IDENTIFICATION BY OTHER LIQUID CHROMATOGRAPHY TECHNIQUES IN CHROMATOGRAPHY (621)

Some of the ions suitable for IC identification can also be identified by other forms of liquid chromatography. Sample and Reference Standard concentrations, as well as injection volumes, need to be adjusted, depending on the detection technique used. All high-concentration components in the test substance should be analyzed to judge whether there is interference with the analyte of interest.

• IDENTIFICATION USING RAMAN SPECTROSCOPY

Raman spectroscopy can be used for identification of counterions. Guidance regarding the use of Raman spectroscopy may be found in Raman Spectroscopy—Theory and Practice (1858) (CN 1-Aug-2020).

Apparatus: Prepare the spectrometer for operation according to the instrument instruction manual and the instrument manufacturer’s recommendations. An instrument performance check and the quality of spectra collected should be evaluated at time of use or following manufacturer’s instructions.

Procedure
Reference standard and Sample: All reference material and sample spectra should be collected using identical instrumental parameters. These instrumental parameters may be determined by the analyst based on the nature of the sample and the type of analysis and should be selected based on the quality of spectra needed. Use the appropriate sample container and/or sample holding apparatus, depending on the type of reference material and sample (powder, liquid, paste, film, or other) being analyzed. Transfer the sample or reference material into the appropriate sample container and/or holder, as needed, and acquire the spectrum for each.

Analysis: Qualitatively compare the Raman spectra obtained from the reference material and the Sample. The Sample complies with the identity test if the spectrum exhibits maxima only at the same wavelengths as that of a similar preparation of the corresponding USP Reference Standard, where available.

• IDENTIFICATION USING MID-INFRARED SPECTROSCOPY

Mid-infrared spectroscopy techniques can be used for identification of counterions. Guidance regarding the use of mid-infrared spectroscopy may be found in Mid-Infrared Spectroscopy (854) and Spectroscopic Identification Tests (197). If the monograph contains an identification test by IR (e.g., (197)) and a reference to (191), then mid-infrared cannot be used as an instrumental replacement for the chemical identification tests prescribed in this chapter.

Procedure
Standard and Sample solutions: Sample preparation may be conducted using any of the procedures described in (854) that are appropriate for the sample of concern. The standard preparation should be conducted in the same way but using the USP Reference Standard of the substance under test, where available.

Analysis: Record the spectra of the test specimen and the corresponding Reference Standard over the range from about 3800 to 650 cm⁻¹ (2.6–13 µm). The IR absorption spectrum of the preparation of the test specimen, previously dried under conditions specified for the corresponding Reference Standard, unless the Reference Standard is to be used without drying,
exhibits maxima only at the same wavelengths as that of a similar preparation of the corresponding USP Reference Standard, where available.

〈197〉 SPECTROSCOPIC IDENTIFICATION TESTS

Change to read:

INTRODUCTION AND SCOPE

This chapter provides several tests and procedures that are used to confirm the chemical identity of a specific material in its respective monograph. When one of the tests listed in this chapter is referenced in a monograph, it must be used to confirm the identity of the material. Alternative identification procedures may be used, provided they are demonstrated to be equivalent to or better than the specified procedure and if they meet the requirements specified in the Equivalent/Alternative Tests section.

Spectroscopic tests contribute meaningfully toward the identification of many official articles. The test procedures that follow are applicable to substances that absorb, transmit, reflect, or scatter electromagnetic radiation in the near-infrared (NIR), infrared (IR), visible or near-infrared (Raman), ultraviolet (UV), visible (Vis), or X-ray ranges (see Mid-Infrared Spectroscopy (854), Ultraviolet-Visible Spectroscopy (857), X-Ray Powder Diffraction (941), Near-Infrared Spectroscopy—Theory and Practice (1856), and Raman Spectroscopy (858)▲(CN 1-Aug-2020)). The NIR, IR, and Raman spectra, or X-ray diffraction pattern of a substance, compared with the spectrum or diffraction pattern obtained with the corresponding USP Reference Standard, provides perhaps the most conclusive evidence of the identity of the substance that can be realized from any single test. The UV or Vis absorption spectrum of a substance, on the other hand, does not exhibit a high degree of specificity in most cases. To provide unambiguous confirmation of the identity of a substance, it may be necessary to execute two (or more) identity tests, as specified in a large proportion of compendial monographs.

IDENTIFICATION METHODOLOGY

Suitable identification methodology must be used for the chemical identification of materials through comparison with the appropriate compendial standards. Where alternative techniques are used, it must be demonstrated that the alternative identification methodology is suitable for the intended application (see Validation of Compendial Procedures (1225)). Identification procedures should be able to discriminate between materials similar in molecular structure. The lack of specificity of a single technique may be compensated by other supporting analytical procedure(s) or an application of an additional identification technique.

The IR spectrum of a substance, compared with that obtained using equivalent instruments and conditions for the corresponding USP Reference Standard, is the most widely used methodology for chemical identification in compendial monographs. In general practice, the analysis of the sample and the USP Reference Standard are completed at the same time; however, if the USP Reference Standard spectrum was obtained previously using equivalent instruments and conditions, it is appropriate to compare the sample spectrum with the stored USP Reference Standard spectrum.

Under conditions where the IR spectrum lacks specificity for definitive chemical identification, additional spectroscopic information can be used to supplement chemical identification. For example, conformance with both IR and UV test specifications, as specified in a large proportion of compendial monographs, provides complementary information for the definitive identity of the sample under examination. In these instances, the combined spectroscopic information enables discrimination between compounds similar in structure that would not be possible from either IR spectrum or UV spectrum alone.

For preparing the standards used in the applications of this chapter, unless the directions for preparing the USP Reference Standard are explicitly specified in the monograph procedure, the USP Reference Standard must be used in accordance with instructions on its label. For preparation of the test sample, the sample must be prepared in accordance with the directions in the individual monograph procedure. Where no specific instructions are provided for sample preparation, handle the sample in the same manner as described by the USP Reference Standard label. For example, dry the sample as per drying conditions on the label of the corresponding USP Reference Standard.

INFRARED SPECTROSCOPY

Several methods are indicated for the preparation of test samples and USP Reference Standards for analysis by infrared spectroscopy (see Table 1 and (854)). The approaches for the techniques used in IR identity testing are summarized in Table 1.

<table>
<thead>
<tr>
<th>Method Reference</th>
<th>Sample Preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>(197A)</td>
<td>The substance under examination is intimately in contact with an internal reflection element for attenuated total reflection (ATR) analysis.</td>
</tr>
<tr>
<td>(197D)</td>
<td>The substance under examination is mixed intimately with potassium bromide and transferred to a sample container for diffuse reflection (DR) analysis.</td>
</tr>
<tr>
<td>(197E)</td>
<td>The substance under examination is pressed as a thin sample against a suitable plate for IR microscopic analysis.</td>
</tr>
</tbody>
</table>
In each instance, infrared spectra of both the sample and corresponding USP Reference Standard are obtained using the same sample preparation technique and measurement parameters. Record and compare the spectra of the sample and the corresponding USP Reference Standard over the range from 3800 to 650 cm\(^{-1}\), unless otherwise specified in the individual monograph. The comparison must establish that the IR spectrum of the preparation of the sample exhibits maxima only at the same wavenumbers as that of the appropriately prepared corresponding USP Reference Standard. If there are differences between the spectra, and the sample spectrum was compared with a previously obtained and electronically stored spectrum of the USP Reference Standard, the comparison must be repeated concomitantly with a freshly prepared USP Reference Standard.

Differences between the USP Reference Standard spectrum and sample spectrum that may be observed are sometimes attributable to differences in the solid-state form of the materials, if a solid-state technique is used (e.g., (197A), (197K), or (197M)). If a specific crystal form is not specified in the monograph, where spectral differences between the sample and USP Reference Standard are observed, recrystallize both the sample and USP Reference Standard under identical conditions to produce the same solid-state form, unless specific procedures are provided in the individual monographs. Dissolve equal portions of the sample and the USP Reference Standard in equal volumes of a suitable solvent, evaporate the solutions to dryness in similar containers under identical conditions, and repeat the identification test on the residues. Other techniques for recrystallizing the sample and USP Reference Standard based on known scientific principles may be used with appropriate scientific justification.

**Change to read:**

**NEAR-INFRARED AND RAMAN SPECTROSCOPY**

The reference (197NIR) in a monograph signifies that a test sample and a standard sample are examined by the NIR spectroscopic technique (see Near-Infrared Spectroscopy—Theory and Practice (1856)), and the reference (197R) in a monograph signifies that a test sample and a standard sample are examined by the Raman spectroscopic technique (see Raman Spectroscopy—Theory and Practice (1858)). NIR and Raman spectroscopic procedures may be used to confirm the identity of materials. The approach is similar to that of IR identity testing but is often augmented through the use of spectral libraries and chemometrics. A sample can thus be analyzed by NIR or Raman techniques, with the resulting spectrum compared with stored spectra in the spectral library through the use of multivariate analysis. In general, a visual comparison or simple overlay of the spectra alone may not be sufficient and additional evaluation may be needed.

In both techniques, samples can be directly interrogated with minimal or no sample preparation. Measurement is nondestructive and noninvasive, and data collection can often be made through glass or plastic containers.

A description of these techniques, and the strategy for procedure and chemometrics model development and validation can be found in the appropriate associated chapters when available (see Chemometrics (1039), Near-Infrared Spectroscopy—Theory and Practice (1856), and Raman Spectroscopy—Theory and Practice (1858)), and the following chapters to be published at a later date: Near-Infrared Spectroscopy—Theory and Practice (1856) and Raman Spectroscopy—Theory and Practice (1858)).

**ULTRAVIOLET-VISIBLE SPECTROSCOPY**

The reference (197U) in a monograph signifies that a sample solution and a Standard solution are examined spectrophotometrically, in 1-cm cells, over the spectral range from 200 to 400 nm, unless otherwise specified in the individual monograph (see (857)). Dissolve a portion of the substance under examination in the designated medium to obtain a sample solution having the concentration specified in the monograph. Record and compare the spectra obtained for the sample solution and the Standard solution. Review or calculate the absorbivities and/or absorbance ratios and compare the results, and where appropriate, compare to criteria specified in an individual monograph.

The comparison must establish that the UV spectrum of the preparation of the sample exhibits absorption maxima and minima only at the same wavelengths as those of the appropriately prepared corresponding USP Reference Standard, and that the absorbivities and/or absorbance ratios are within the specified limits. If there are differences in the spectra, and the sample spectrum was compared with a previously obtained and electronically stored spectrum of the USP Reference Standard, the comparison must be concomitantly repeated with a freshly prepared USP Reference Standard.

Unless otherwise specified in the monograph, absorbances indicated for the calculations of the absorbivities and/or absorbance ratios are those measured at the maximum absorbance wavelength (within ±2 nm) specified in the individual
monograph. Where the absorbance is to be measured at about the specified wavelength other than that of maximum absorbance, the abbreviations for minimum (min) and shoulder (sh) are used, respectively, in an absorption spectrum.

The requirements are met if the UV spectra of the sample solution and of the Standard solution exhibit maxima and minima at the same wavelengths, and, if applicable, the absorptivities and/or absorbance ratios are within specified limits.

**X-RAY POWDER DIFFRACTION**

The reference (197XR) in a monograph signifies that a test sample and a standard sample are examined according to (941). Prepare and mount the specimen as directed in (941). Unless otherwise indicated in the monograph, record the diffraction pattern in a 2θ-range from as near to 0° as possible to at least 32°. Unless otherwise specified in the monograph, the requirements are met if the X-ray diffraction pattern of the test specimen conforms to that of the corresponding USP Reference Standard obtained using equivalent instruments and conditions. Differences in the diffraction line intensities (but not line positions) between the sample and the Standard are acceptable.

The comparison must establish that the diffraction pattern of the preparation of the test specimen conforms to the diffraction pattern of the corresponding USP Reference Standard. If there are differences in the diffraction pattern and the sample diffraction pattern was compared with a previously obtained and electronically stored diffraction pattern of the USP Reference Standard, the comparison must be repeated concomitantly with a freshly prepared USP Reference Standard.

**EQUIVALENT/ALTERNATIVE TESTS**

In addition to IR, NIR, Raman, X-ray, and UV absorption, several other spectroscopic methodologies can be utilized for the identification of the specimen under examination. The methods cited in this chapter may be used for identification of materials as an alternative method to the method referenced in the monograph, provided that the alternative technique has been determined to be suitable for identification. Suitable identification tests must be able to discriminate between compounds similar in molecular structure that are likely to be present. The choice of such potentially interfering materials must be based on sound scientific judgment, with consideration of interferences that could occur. It is not always possible to demonstrate that an analytical procedure is specific for a particular analyte (complete discrimination).

For information regarding sample preparation and measurement parameters associated with an alternative identification method, refer to the appropriate general chapter (see Mass Spectrometry (736), Nuclear Magnetic Resonance Spectroscopy (761), (854), (857), (941), Near-Infrared Spectroscopy—Theory and Practice (1856), and ▲(858)▲ (CN 1-Aug-2020).

**THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**

**PROCEDURES**

- **General Procedure**
  The following procedure is applicable as an aid in verifying the identities of many compendial drug substances as such and in their respective dosage forms.
  
  Prepare a test solution as directed in the individual monograph. On a line parallel to and about 2 cm from the edge of a suitable thin-layer chromatographic plate, coated with a 0.25-mm layer of chromatographic silica gel mixture (see Chromatography (621)) apply 10 µL of this solution and 10 µL of a Standard solution prepared from the USP Reference Standard for the drug substance being identified, in the same solvent and at the same concentration as the test solution, unless otherwise directed in the individual monograph. Allow the spots to dry, and develop the chromatogram in a solvent system consisting of a mixture of chloroform, methanol, and water (180:15:1), unless otherwise directed in the individual monograph, until the solvent front has moved about three-fourths of the length of the plate. Remove the plate from the developing chamber, mark the solvent front, and allow the solvent to evaporate. Unless otherwise directed in the individual monograph, locate the spots on the plate by examination under short-wavelength UV light. The Rf value of the principal spot obtained from the test solution corresponds to that obtained from the Standard solution.

- **Procedure for Bacitracin, Neomycin, and Polymyxin B**
  The following thin-layer chromatographic procedure is applicable as an aid in verifying the identities of bacitracin, neomycin, and polymyxin B active ingredients and in dosage forms when present singly and in two- and three-component mixtures.
  
  Prepare a Test solution as follows, unless otherwise directed in the individual monograph.

  **Test solution**
  
  **For drug substances:** Dissolve a portion of Bacitracin, Bacitracin Zinc, Neomycin Sulfate, or Polymyxin B Sulfate in 0.1 N hydrochloric acid to obtain a solution containing about 500 USP Bacitracin Units per mL, 3.5 mg of neomycin (base) per mL, or 10,000 USP Polymyxin B Units per mL.

  **For solutions:** Where the solution contains neomycin and polymyxin B, dilute a portion of it with 0.1 N hydrochloric acid to obtain a solution containing the equivalent of about 3.5 mg of neomycin (base) per mL. Where the Solution contains polymyxin B but not neomycin, dilute a portion of it with 0.1 N hydrochloric acid to obtain a solution containing about 10,000 USP Polymyxin B Units per mL.
For creams, lotions, and ointments: Where the Cream, Lotion, or Ointment contains Bacitracin or Bacitracin Zinc, transfer a portion of it equivalent to about 500 USP Bacitracin Units, to a 15-mL centrifuge tube. Where the Cream, Lotion, or Ointment contains neomycin, but not Bacitracin or Bacitracin Zinc, transfer a portion of it equivalent to about 3.5 mg of neomycin (base) per mL to a 15-mL centrifuge tube. Add 4 mL of chloroform to the centrifuge tube, and shake well to disperse the Cream, Lotion, or Ointment. Add 1 mL of 0.1 N hydrochloric acid, vortex for 4 minutes, centrifuge, and use the clear supernatant.

[NOTE—The Modified test solution as described below in the Modified procedure may be used in lieu of the Test solution.]

**Standard bacitracin solution:** Dissolve a portion of USP Bacitracin Zinc RS in 0.1 N hydrochloric acid to obtain a solution containing 500 USP Bacitracin Units per mL.

**Standard neomycin solution:** Dissolve a portion of USP Neomycin Sulfate RS in 0.1 N hydrochloric acid to obtain a solution containing the equivalent of 3.5 mg of neomycin (base) per mL.

**Standard polymyxin B solution:** Dissolve a portion of USP Polymyxin B Sulfate RS in 0.1 N hydrochloric acid to obtain a solution containing 10,000 USP Polymyxin B Units per mL. Where the article under test also contains Bacitracin or Bacitracin Zinc, dissolve a portion of USP Polymyxin B Sulfate RS in 0.1 N hydrochloric acid to obtain a solution containing 500/USP Polymyxin B Units per mL, J being the ratio of the labeled amount of USP Polymyxin B Units to the labeled amount of USP Bacitracin Units in each g of Cream, Lotion, or Ointment.

**Developing solvent solution:** Prepare a mixture of methanol, isopropyl alcohol, methylene chloride, ammonium hydroxide, and water (4:2: 2: 1.5).

**Procedure:** Apply 10 µL of the Test solution and each of the relevant Standard solutions to a suitable thin-layer chromatographic plate (see Chromatography (621)) coated with a 0.25-mm layer of chromatographic silica gel. Place the plate in a presaturated chromatographic chamber, and develop the chromatogram with the Developing solvent system until the solvent front has moved about three-fourths of the length of the plate. Remove the plate from the chamber, and dry at 105° for 10 minutes. Spray the plate with a 0.2% solution of ninhydrin in butyl alcohol, and heat at 105° for 5 minutes. The Rf value of each principal spot in the chromatogram of the Test solution corresponds to that of the principal spot in the chromatogram obtained from each relevant Standard solution as appropriate for the labeled active ingredient or ingredients specified on the label. If the chromatogram of the Test solution yields excessive streaking, proceed as directed for Modified procedure.

**Modified procedure:** Transfer the Test solution to a 15-mL centrifuge tube, add 10 mL of saturated aqueous picric acid solution (1.2%, w/v), vortex for 1 minute, centrifuge for 10 minutes, and discard the supernatant. Wash the residue with 1-mL portions of water until no yellow color is observed in the washing. Discard the washings, and dry the residue under a stream of nitrogen at 50°. Dissolve the residue in 1 mL of acetone, add 1 mL of a freshly prepared solution of sulfuric acid in acetone (1 in 100), shake, centrifuge for 5 minutes, and discard the supernatant. Rinse the residue with 1 mL of acetone, centrifuge briefly, and discard the washing. Repeat the washing until no yellow color is observed. Dry the residue under a stream of nitrogen at 50°. Dissolve the residue in 0.5 mL of 0.1 N hydrochloric acid (Modified test solution). Repeat the Procedure using this Modified test solution instead of the Test solution. The Rf value of each principal spot in the chromatogram of the Modified test solution corresponds to that of the principal spot in the chromatogram obtained from each relevant Standard solution as appropriate for the active ingredient or ingredients specified on the label.

**USP Reference Standards** (11)

USP Bacitracin Zinc RS
USP Neomycin Sulfate RS
USP Polymyxin B Sulfate RS

### ALUMINUM

This procedure is provided to demonstrate that the content of aluminum (Al) does not exceed the limit given in the individual monograph of a substance labeled as intended for use in hemodialysis. [NOTE—The Standard Preparations and the Test Preparation may be modified, if necessary, to obtain solutions of suitable concentrations adaptable to the linear or working range of the instrument.]

**NITRIC ACID DILUENT**

Transfer 40 mL of nitric acid to a 1000-mL volumetric flask, and dilute with water to volume.

**STANDARD PREPARATIONS**

Treat some aluminum wire with 6 N hydrochloric acid at 80° for a few minutes. Dissolve about 100 mg of the treated wire, accurately weighed, in a mixture of 10 mL of hydrochloric acid and 2 mL of nitric acid by heating at about 80° for approximately 30 minutes. Continue heating until the volume is reduced to about 4 mL. Cool to room temperature, and add 4 mL of water. Evaporate to about 2 mL by heating. Cool, and transfer this solution, with the aid of water, to a 100-mL volumetric flask, dilute with water to volume, and mix. Transfer 10.0 mL of this solution to a second 100-mL volumetric flask, dilute with water to volume, and mix. Transfer 1.0 mL of this solution to a third 100-mL volumetric flask, dilute with water to volume, and mix. The concentration of aluminum in this Standard Preparation is about 1.0 µg per mL. If a more diluted Standard Preparation is required, transfer 1.0-, 2.0-, and 4.0-mL portions of this solution to separate 100-mL volumetric flasks, dilute with Nitric Acid Diluent to volume, and mix. These solutions contain 0.01, 0.02, and 0.04 µg of Al per mL, respectively.
**TEST PREPARATION**

Unless otherwise directed in the monograph, transfer an accurately weighed amount (in g) of the test substance, as specified in the monograph, to a 100-mL plastic volumetric flask, add 50 mL of water, and sonicate for 30 minutes. Add 4 mL of nitric acid, dilute with water to volume, and mix.

**PROCEDURE**

Determine the absorbances of the Standard Preparations and the Test Preparation at the aluminum emission line at 309.3 nm with a suitable atomic absorption spectrophotometer (see Atomic Absorption Spectroscopy (852)) equipped with an aluminum hollow-cathode lamp and a flameless electrically heated furnace, using the Nitric Acid Diluent as the blank. Plot the absorbances of the Standard Preparations versus the content of Al, in µg per mL, drawing a straight line best fitting the three points. From the graph so obtained, determine the quantity, in µg, of Al in each mL of the Test Preparation. Calculate the amount of Al in the specimen taken, in µg per g, by multiplying this value by 100/W, where W is the weight, in g, of the substance taken to prepare the Test Preparation.

### (221) CHLORIDE AND SULFATE

**Change to read:**

The following limit tests are provided as general procedures for use where limits for chloride and sulfate are specified in the individual monographs.

Perform the tests and the controls in glass cylinders of the same diameter and matched as closely as practicable in other respects (see **Visual Comparison** (630)(CN 1-May-2019)). Use the same quantities of the same reagents for both the solution under test and the control solution containing the specified volume of chloride or sulfate. If, after acidification, the solution is not perfectly clear, pass it through a filter paper that gives negative tests for chloride and sulfate. Add the precipitant, silver nitrate TS or barium chloride TS as required, to both the test solution and the control solution in immediate sequence. Where the individual monograph calls for applying the test to a specific volume of a solution of the substance, and the limit for chloride or sulfate corresponds to 0.20 mL or less of 0.020 N hydrochloric acid or sulfuric acid, respectively, apply the test to the solution without further dilution. In such cases maintain the same volume relationships for the control solution as specified for the solution under test. In applying the test to the salts of heavy metals, which normally show an acid reaction, omit the acidification and do not neutralize the solution. Dissolve bismuth salts in a few mL of water and 2 mL of nitric acid before treating with the precipitant.

**CHLORIDE**—Dissolve the specified quantity of the substance under test in 30 to 40 mL of water, or, where the substance is already in solution, add water to make a total volume of 30 to 40 mL, and, if necessary, neutralize the solution with nitric acid to litmus. Add 1 mL each of nitric acid and of silver nitrate TS and sufficient water to make 50 mL. Mix, and allow to stand for 5 minutes protected from direct sunlight. Unless otherwise specified in the monograph, compare the turbidity, if any, with that produced in a solution containing the volume of 0.020 N hydrochloric acid specified in the monograph.

**SULFATE**—Dissolve the specified quantity of the substance under test in 30 to 40 mL of water, or, where the substance is already in solution, add water to make a total volume of 30 to 40 mL, and, if necessary, neutralize the solution with hydrochloric acid to litmus. Add 1 mL of 3 N hydrochloric acid, 3 mL of barium chloride TS, and sufficient water to make 50 mL. Mix, and allow to stand for 10 minutes. Unless otherwise specified in the monograph, compare the turbidity, if any, with that produced in a solution containing the volume of 0.020 N sulfuric acid specified in the monograph.

### (281) RESIDUE ON IGNITION

Portions of this general chapter have been harmonized with the corresponding texts of the European Pharmacopoeia and the Japanese Pharmacopoeia. The portions that are not harmonized are marked with symbols (*,*). The harmonized texts of these pharmacopeias are therefore interchangeable, and the methods of the European Pharmacopoeia and/or the Japanese Pharmacopoeia may be used for demonstration of compliance instead of the present United States Pharmacopoeia general chapter. These pharmacopeias have undertaken not to make any unilateral change to this harmonized chapter.

The Residue on Ignition/Sulfated Ash test uses a procedure to measure the amount of residual substance not volatilized from a sample when the sample is ignited in the presence of sulfuric acid according to the procedure described below. This test is usually used for determining the content of inorganic impurities in an organic substance.

**PROCEDURE**

Ignite a suitable crucible (for example, silica, platinum, quartz, or porcelain) at 600 ± 50° for 30 minutes, cool the crucible in a desiccator (silica gel or other suitable desiccant), and weigh it accurately. Weigh accurately *1 to 2 g of the substance, or, the amount specified in the individual monograph, in the crucible.

Moisten the sample with a small amount (usually 1 mL) of sulfuric acid, then heat gently at a temperature as low as practicable until the sample is thoroughly charred. Cool; then,* unless otherwise directed in the individual monograph,* moisture the residue
with a small amount (usually 1 mL) of sulfuric acid; heat gently until white fumes are no longer evolved; and ignite at 600 ± 50°, unless another temperature is specified in the individual monograph, until the residue is completely incinerated. Ensure that flames are not produced at any time during the procedure. Cool the crucible in a desiccator (silica gel or other suitable desiccant), weigh accurately, and calculate the percentage of residue.

Unless otherwise specified, if the amount of the residue so obtained exceeds the limit specified in the individual monograph, repeat the moistening with sulfuric acid, heating and igniting as before, using a 30-minute ignition period, until two consecutive weighings of the residue do not differ by more than 0.5 mg or until the percentage of residue complies with the limit in the individual monograph.

*Conduct the ignition in a well-ventilated hood, but protected from air currents, and at as low a temperature as is possible to effect the complete combustion of the carbon. A muffle furnace may be used, if desired, and its use is recommended for the final ignition at 600 ± 50°.

Calibration of the muffle furnace may be carried out using an appropriate digital temperature meter and a working thermocouple probe calibrated against a standard thermocouple traceable to the National Institute of Standards and Technology.

Verify the accuracy of the measuring and controlling circuitry of the muffle furnace by checking the positions in the furnace at the control set point temperature of intended use. Select positions that reflect the eventual method of use with respect to location of the specimen under test. The tolerance is ±25° at each position measured.

〈341〉 ANTIMICROBIAL AGENTS—CONTENT

An essential component of Injections preserved in multiple-dose containers is the antimicrobial agent or antimicrobial agents present to reduce the hazard of having introduced, in the course of removing some of the contents, accidental microbial contamination of the contents remaining. It is a Pharmacopeial requirement that the presence and amount added of such antimicrobial agent(s) be declared on the label of the container. This general chapter provides methods for the most commonly used antimicrobial agents. These methods or other suitably validated methods are to be used to demonstrate that the declared antimicrobial agent is present but does not exceed the labeled amount by more than 20%.

The concentration of an antimicrobial preservative added to a multiple-dose or single-dose parenteral, otic, nasal, and ophthalmic preparation may diminish during the shelf life of the product. Therefore, the manufacturer shall determine the lowest level at which the preservative is effective, and the product should be so formulated as to assure that this level is exceeded throughout the product’s shelf life. At the time of its manufacture, the product should contain the declared amount of antimicrobial preservative (within ±20% to allow for manufacturing and analytical variations). The quantitative label statement of the preservative content is not intended to mean that the labeled quantity is retained during the shelf life of the product; rather, it is a statement of the amount added, within process limits, and which is not exceeded by more than 20%. An example of such a label statement is “____(unit) added as preservative”. [NOTE—“____(unit)” would be a number followed by the unit of measurement, e.g., 0.015 mg/mL or 0.1%.

The most commonly used antimicrobial agents include benzyl alcohol; chlorobutanol; phenol; the four homologous esters of p-hydroxybenzoic acid (methyl, ethyl, propyl, and butyl parabens); and the two mercurials, phenylmercuric nitrate and thimerosal. The method used for phenylmercuric nitrate is polarographic, whereas quantitative liquid chromatography was used for thimerosal and the four homologous esters of p-hydroxybenzoic acid. Gas chromatography is used in the determination of phenol, benzyl alcohol, and chlorobutanol.

GENERAL GAS AND LIQUID CHROMATOGRAPHIC METHODS

The general gas chromatography procedures set forth in the following paragraphs are applicable to the quantitative determination of benzyl alcohol, chlorobutanol, and phenol. Prepare the Internal standard solution and the Standard solution for each antimicrobial agent as directed below. Unless otherwise directed by the individual monograph, prepare the Sample solution from accurately measured portions of the sample under test and the Internal standard solution such that the concentration of the antimicrobial agent and the composition of the solvent correspond closely to the concentration and composition of the Standard solution. Suggested operating parameters of the gas chromatograph are provided in this section.

The general high-pressure liquid chromatography (HPLC) procedures set forth in the following paragraphs are applicable to the quantitative determination of parabens and Thimerosal. Prepare the Internal standard solution and the Standard solution for each antimicrobial agent as directed below. Unless otherwise directed, prepare the Sample solution from accurately measured portions of the sample under test and the Internal standard solution, if applicable, such that the concentration of the antimicrobial agent and the composition of the solvent is about the same as the concentration and composition of the Standard solution. Suggested operating parameters of the liquid chromatograph are provided in this section.

- BENZYL ALCOHOL
  Diluent: Methanol and water (20:80)
  Internal standard solution: 3.8 mg/mL of phenol prepared as follows. Dissolve a suitable amount of phenol in 10% of the flask volume of methanol, and dilute with water to volume.
  Standard solution: 1.8 mg/mL of USP Benzyl Alcohol RS and 1.5 mg/mL of phenol prepared as follows. Dissolve 180 mg of USP Benzyl Alcohol RS in 20 mL of methanol contained in a 100-mL volumetric flask. Add 40.0 mL of Internal standard solution, and dilute with water to volume.
  Chromatographic system
  (See Chromatography (621), System Suitability.)
  Mode: GC
  Detector: Flame ionization
  Column: 30-m × 0.32-mm fused-silica; bonded with a 0.5-µm film of phase G16

Published on March 26, 2020
Temperatures
Injection port: 200°
Detector: 310°
Column: See Table 1.

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<thead>
<tr>
<th></th>
<th>Initial Temperature (°)</th>
<th>Temperature Ramp (°/min)</th>
<th>Final Temperature (°)</th>
<th>Hold Time at Final Temperature (min)</th>
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</table>

Carrier gas: Helium
Flow rate (constant): 2 mL/min
Injection volume: 1 µL
Split ratio: 10:1
Run time: 20 min

System suitability
Sample: Standard solution

Suitability requirements
Resolution: NLT 2.0 between the benzyl alcohol and phenol peaks
Tailing factor: NMT 2.0 for the benzyl alcohol peak
Relative standard deviation: NMT 2.0% for the peak response ratio of benzyl alcohol to phenol

Analysis
Samples: Standard solution and Sample solution
Calculate the percentage of the labeled amount of benzyl alcohol (C₇H₈O) in the portion of the sample taken:

\[
\text{Result} = \left( \frac{R_U}{R_S} \right) \times \left( \frac{C_S}{C_U} \right) \times 100
\]

\[
R_U = \text{peak response ratio of benzyl alcohol to phenol from the Sample solution}
\]

\[
R_S = \text{peak response ratio of benzyl alcohol to phenol from the Standard solution}
\]

\[
C_S = \text{concentration of USP Benzyl Alcohol RS in the Standard solution}
\]

\[
C_U = \text{nominal concentration of benzyl alcohol in the Sample solution}
\]

Chlorobutanol
Diluent: Methanol and water (50:50)
Internal standard solution: 10 mg/mL of 2,2,2-trichloroethanol in Diluent
Standard stock solution: 5 mg/mL of USP Chlorobutanol RS in methanol
Standard solution: 1.25 mg/mL of USP Chlorobutanol RS and 2 mg/mL of 2,2,2-trichloroethanol prepared as follows. Transfer 2.5 mL of Standard stock solution, 2.0 mL of Internal standard solution, and 0.5 mL of methanol to a 10-mL volumetric flask. Dilute with water to volume.
Sample stock solution: Quantitatively dilute, if necessary, an accurately measured volume corresponding to 2.5 mg/mL of chlorobutanol in water.
Sample solution: Combine 5.0 mL of Sample stock solution with 2.0 mL of Internal standard solution in a 10-mL volumetric flask, and dilute with Diluent to volume.

Chromatographic system
(See Chromatography (621), System Suitability.)
Mode: GC
Detector: Flame ionization
Column: 30-m × 0.32-mm fused-silica; bonded with a 0.25-µm film of phase G16
Temperatures
Injection port: 260°
Detector: 280°
Column: 135°
Carrier gas: Helium
Flow rate: 1 mL/min
Injection volume: 0.5 µL
Split ratio: 10:1
Run time: 12 min

System suitability
Sample: Standard solution

Suitability requirements
Resolution: NLT 2.0 between chlorobutanol and 2,2,2-trichloroethanol
Relative standard deviation: NMT 1.0% for the peak response ratio of chlorobutanol to 2,2,2-trichloroethanol

Analysis
Samples: Standard solution and Sample solution
Calculate the percentage of the labeled amount of chlorobutanol \((\text{C}_4\text{H}_7\text{Cl}_3\text{O})\), on the anhydrous basis, in the portion of the sample taken:

\[
\text{Result} = \left(\frac{R_U}{R_S}\right) \times \left(\frac{C_S}{C_U}\right) \times 100
\]

- \(R_U\) = peak response ratio of chlorobutanol to 2,2,2-trichloroethanol from the Sample solution
- \(R_S\) = peak response ratio of chlorobutanol to 2,2,2-trichloroethanol from the Standard solution
- \(C_S\) = concentration of USP Chlorobutanol RS in the Standard solution
- \(C_U\) = nominal concentration of chlorobutanol in the Sample solution

**PHENOL**

- **Internal standard solution**: 2 mg/mL of USP Benzyl Alcohol RS in methanol
- **Standard stock solution**: 4 mg/mL of USP Phenol RS in water
- **Standard solution**: 0.4 mg/mL each of USP Phenol RS and USP Benzyl Alcohol RS prepared as follows. Combine 5.0 mL of **Standard stock solution** with 10.0 mL of **Internal standard solution** in a 50-mL volumetric flask, and dilute with water to volume.

**Chromatographic system**

- **Mode**: GC
- **Detector**: Flame ionization
- **Column**: 30-m × 0.32-mm fused-silica; bonded with a 0.5-µm film of phase G16
- **Temperatures**
  - Injection port: 200°
  - Detector: 310°
  - Column: See Table 2.

<table>
<thead>
<tr>
<th>Initial Temperature (°)</th>
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- **Carrier gas**: Helium
- **Flow rate (constant flow)**: 2 mL/min
- **Injection volume**: 1 µL
- **Split ratio**: 10:1
- **Run time**: 20 min

**System suitability**

- **Sample**: Standard solution
  - [Note—The relative retention times for benzyl alcohol and phenol are about 0.85 and 1.0, respectively.]

**Suitability requirements**

- **Resolution**: NLT 2.0 between benzyl alcohol and phenol
- **Tailing factor**: NMT 2.0 for the phenol peak
- **Relative standard deviation**: NMT 1.0% for the peak response ratio of phenol to benzyl alcohol

**Analysis**

- **Samples**: Standard solution and Sample solution

Calculate the percentage of the labeled amount of phenol \((\text{C}_6\text{H}_6\text{O})\) in the portion of the sample taken:

\[
\text{Result} = \left(\frac{R_U}{R_S}\right) \times \left(\frac{C_S}{C_U}\right) \times 100
\]

- \(R_U\) = peak response ratio of phenol to benzyl alcohol from the Sample solution
- \(R_S\) = peak response ratio of phenol to benzyl alcohol from the Standard solution
- \(C_S\) = concentration of USP Phenol RS in the Standard solution
- \(C_U\) = nominal concentration of phenol in the Sample solution

**METHYLPARABEN AND PROPYLPARABEN**

- **Buffer**: 7 g/L of monobasic potassium phosphate in water
- **Mobile phase**: Methanol and Buffer (65:35)
- **Internal standard solution**: 0.013 mg/mL of USP Ethylparaben RS in Mobile phase
- **System suitability solution**: 0.01 mg/mL each of USP Butylparaben RS, USP Propylparaben RS, USP Ethylparaben RS, USP Methylparaben RS, and p-hydroxybenzoic acid in Mobile phase
- **Standard stock solution**: 0.2 mg/mL of USP Methylparaben RS and 0.03 mg/mL of USP Propylparaben RS in Mobile phase
- **Standard solution**: Combine 5 mL of **Standard stock solution** with 5 mL of **Internal standard solution**, and extract three times with 10-mL aliquots of diethyl ether. Filter the combined ether layers through anhydrous sodium sulfate. Evaporate the ether extract to dryness, and dissolve the residue in 50 mL of Mobile phase.
Sample solution: Combine 5 mL of the specimen under test with 5 mL of *Internal standard solution*, and extract three times with 10-mL aliquots of diethyl ether. Filter the combined ether layers through anhydrous sodium sulfate. Evaporate the ether extract to dryness, and dissolve the residue in 50 mL of *Mobile phase*.

Chromatographic system
(See *Chromatography (621)*, *System Suitability*.)
Mode: LC
Detector: UV 272 nm
Columns
Guard: 4.0-mm × 3-mm; packing L1
Analytical: 4.6-mm × 15-cm; 5-µm packing L1
Flow rate: 1.3 mL/min
Injection volume: 10 µL
Run time: 10 min

System suitability
Samples: *System suitability solution* and *Standard solution*
[NOTE—The relative retention times for *p*-hydroxybenzoic acid, methylparaben, ethylparaben, and propylparaben are about 0.58, 1.0, 1.4, and 2.1, respectively.]

Suitability requirements
Resolution: NLT 2.0 between *p*-hydroxybenzoic acid and methylparaben, NLT 2.0 between methylparaben and ethylparaben; *System suitability solution*
Tailing factor: NMT 2.0 for the methylparaben and propylparaben peaks, *Standard solution*
Relative standard deviation: NMT 2.0% for the peak response ratio of methylparaben to ethylparaben, NMT 2.0% for the peak response ratio of propylparaben to ethylparaben; *System suitability solution*

Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of the labeled amount of methylparaben (*C₈H₈O₃*) in the portion of the sample taken:

\[
\text{Result} = \left( \frac{R_U}{R_S} \right) \times \left( \frac{C_S}{C_U} \right) \times 100
\]

\(R_U\) = peak response ratio of methylparaben to ethylparaben from the *Sample solution*
\(R_S\) = peak response ratio of methylparaben to ethylparaben from the *Standard solution*
\(C_S\) = concentration of USP Methylparaben RS in the *Standard solution*
\(C_U\) = nominal concentration of methylparaben in the *Sample solution*

Calculate the percentage of the labeled amount of propylparaben (*C₁₀H₁₂O₃*) in the portion of the sample taken:

\[
\text{Result} = \left( \frac{R_U}{R_S} \right) \times \left( \frac{C_S}{C_U} \right) \times 100
\]

\(R_U\) = peak response ratio of propylparaben to ethylparaben from the *Sample solution*
\(R_S\) = peak response ratio of propylparaben to ethylparaben from the *Standard solution*
\(C_S\) = concentration of USP Propylparaben RS in the *Standard solution*
\(C_U\) = nominal concentration of propylparaben in the *Sample solution*

Ethylparaben and butylparaben may be determined in a similar manner using appropriate internal standard solutions. However, because the extraction recovery is matrix dependent, the user should verify the suitability of the procedure for their drug product and for different product formulations.

**THIMEROSAL**
Solution A: Trifluoroacetic acid and water (0.5: 1000)
Mobile phase: Methanol and *Solution A* (60:40)
Standard solution: 25 µg/mL of USP Thimerosal RS in water
Chromatographic system
(See *Chromatography (621)*, *System Suitability*.)
Mode: LC
Detector: UV 222 nm
Column: 2.1-mm × 10-cm; 2-µm packing L1
Autosampler temperature: 4°C
Flow rate: 0.35 mL/min
Injection volume: 2.5 µL

System suitability
Sample: *Standard solution*
Tailing factor: NMT 1.5
Relative standard deviation: NMT 1.0%

Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of the labeled amount of thimerosal (*C₉H₉HgNaO₂S*) in the portion of the sample taken:

\[
\text{Result} = \left( \frac{r_U}{r_S} \right) \times \left( \frac{C_S}{C_U} \right) \times 100
\]

\(r_U\) = peak response of thimerosal from the *Sample solution*
ORDINARY IMPURITIES

This test, where called for in the individual monograph, is provided to evaluate the presence of ordinary impurities in official articles. Ordinary impurities are defined as those species in drug substances and/or drug products that have no significant, undesirable biological activity in the amounts present. These impurities may arise out of the synthesis, preparation, or degradation of compendial articles. In certain instances, impurities that pose a potential health risk may be detected. Because these impurities would not be individually identified by the strict use of this General Chapter, a separate evaluation may be necessary to ensure that the detected impurities fit the requirements set forth in the definition of Ordinary Impurities. Selections of tests and assays allow for anticipated amounts of impurities that are unobjectionable for the customary use of the article.

REPORTING AND SPECIFICATIONS

The value of 2.0%, unless otherwise specified in the individual monograph, was selected as the general limit for the total amount of ordinary impurities in monographs where documentation did not support adoption of other values.

Where a monograph sets limits on concomitant components and/or specified impurities/degradation products, these species are not to be included in the estimation of ordinary impurities unless so stated in the individual monograph. Concomitant components are defined as species characteristic of many drug substances that are not considered to be impurities in the Pharmacopeial sense. Examples of concomitant components are geometric and optical isomers (or racemates) and antibiotics that are mixtures. Any component that can be considered a toxic impurity because of significant undesirable biological effect is not considered to be a concomitant component.

METHODOLOGY

Unless otherwise specified in an individual monograph, estimation of the amount and number of ordinary impurities is made by relative methods rather than by strict comparison to individual Reference Standards. Nonspecific detection of ordinary impurities is also consistent with this classification.

Typical evaluation methods used for ordinary impurities are thin-layer chromatographic (TLC) techniques. See Chromatography (621) for a general discussion of the thin-layer chromatographic technique. Tests for related substances or chromatographic purity may also be used to evaluate the presence of ordinary impurities. Other methods (e.g., HPLC, HPTLC,
etc.) may also be used with adequate justification as an alternate method. Unless otherwise specified in the individual monograph, use the following method.

**Test Solution**

Prepare, in the solvent specified in the monograph, a solution of the substance under test having an accurately known final concentration of about 10 mg per mL. [Note—Heat or sonication may be used to dissolve the drug substance where use of such does not adversely affect the compound.]

**Standard Solutions**

Prepare, in the solvent specified in the monograph, solutions of the USP Reference Standard or designated substance having accurately known concentrations of 0.01 mg per mL, 0.05 mg per mL, 0.1 mg per mL, and 0.2 mg per mL. [Note—Heat or sonication may be used to dissolve the drug substance where use of such does not adversely affect the compound.]

**Procedure**

Use a thin-layer chromatographic plate coated with a 0.25-mm layer of chromatographic silica gel mixture, and the *Eluant* specified in the monograph. Apply equal volumes (20 µL) of the *Test Solution* and *Standard Solutions* to the plate, using a stream of nitrogen to dry the spots. Allow the chromatogram to develop in a pre-equilibrated chamber until the solvent front has moved about three-fourths of the length of the plate. Remove the plate from the chamber, and air-dry. View the plate using the visualization technique(s) specified. Locate any spots other than the principal spot in the chromatogram of the *Test Solution*, and determine their relative intensities by comparison with the chromatograms of the appropriate *Standard Solutions*. See discussion above with regard to reporting and specifying total ordinary impurities.

**Change to read:**

**KEY FOR VISUALIZATION TECHNIQUES**

1. Use UV light at 254 nm and at about 366 nm.
2. Use Iodoplatinate TS.
3. **Solution A**—Mix 850 mg of bismuth subnitrate with 40 mL of water and 10 mL of glacial acetic acid. **Solution B**—Dissolve 8 g of potassium iodide in 20 mL of water. Mix A and B together to obtain a Stock Solution which can be stored for several months in a dark bottle. Mix 10 mL of the Stock Solution with 20 mL of glacial acetic acid, and dilute with water to make 100 mL, to prepare the spray reagent.
4. **Ninhydrin Spray**—Dissolve 200 mg of ninhydrin in 100 mL of alcohol. Heat the plate after spraying.
5. **Acid Spray**—In an ice bath, add slowly and cautiously, with stirring, 10 mL of sulfuric acid to 90 mL of alcohol. Spray the plate, and heat until charred.
6. **Acid–Dichromate Spray**—Add sufficient potassium dichromate to 100 mL of sulfuric acid to make a saturated solution. Spray the plate, and heat until charred.
7. **Vanillin**—Dissolve 1 g of vanillin in 100 mL of sulfuric acid.
8. **Chloramine T–Trichloroacetic Acid**—Mix 10 mL of a 3% aqueous solution of chloramine T with 40 mL of a 25% alcoholic solution of trichloroacetic acid. Prepare immediately before use.
9. **Folin–C**—Add 10 g of sodium tungstate and 2.5 g of sodium molybdate to 70 mL of water, add 5 mL of 85% phosphoric acid and 10 mL of 36% hydrochloric acid, and reflux this solution for 10 hours.
10. **KMnO₄**—Dissolve 100 mg of Potassium Permanganate in 100 mL of water.
11. **DAB**—Mix 1 g of *p*-dimethylaminobenzaldehyde in 100 mL of 0.6 N hydrochloric acid.
12. **DAC**—Mix 100 mg of *p*-dimethylaminocinnamaldehyde in 100 mL of 1 N hydrochloric acid.
13. **Ferricyanide**—Mix equal volumes of a 1% ferric chloride solution and a 1% potassium ferricyanide solution. Use immediately.
14. **Fast Blue B**—Reagent A—Dissolve 500 mg of Fast Blue B Salt in 100 mL of water. Reagent B—0.1 N sodium hydroxide. Spray first with A, then with B.
15. **Alkaline Ferric Cyanide**—Dilute 1.5 mL of a 1% potassium ferricyanide solution with water to 20 mL, and add 10 mL of 15% sodium hydroxide solution.
16. **Iodine Spray**—Prepare a 0.5% solution of iodine in chloroform.
17. Expose the plate for 10 minutes to iodine vapors in a pre-equilibrated closed chamber, on the bottom of which there are iodine crystals.
18. **Solution A**—Dissolve 0.5 g of potassium iodide in 50 mL of water. **Solution B**—Prepare a solution of 0.5 g of soluble starch in 50 mL of hot water.

Just prior to use, mix equal volumes of *Solution A* and *Solution B*. **Published on March 26, 2020**
DIRECT TITRATIONS

Direct titration is the treatment of a soluble substance, contained in solution in a suitable vessel (the titrate), with an appropriate standardized solution (the titrant), the endpoint being determined instrumentally or visually with the aid of a suitable indicator.

The titrant is added from a suitable buret and is so chosen, with respect to its strength (normality), that the volume added is between 30% and 100% of the rated capacity of the buret. [Note—Where less than 10 mL of titrant is required, a suitable microburet is to be used.]

The endpoint is approached directly but cautiously, and finally the titrant is added dropwise from the buret in order that the final drop added will not overrun the endpoint. The quantity of the substance being titrated may be calculated from the volume and the normality or molarity factor of the titrant and the equivalence factor for the substance given in the individual monograph.

RESIDUAL TITRATIONS

Some Pharmacopeial assays require the addition of a measured volume of a volumetric solution, in excess of the amount actually needed to react with the substance being assayed, the excess of this solution then being titrated with a second volumetric solution. This constitutes a residual titration and is known also as a “back titration.” The quantity of the substance being titrated may be calculated from the difference between the volume of the volumetric solution originally added, corrected by means of a blank titration, and that consumed by the titrant in the back titration, due allowance being made for the respective normality or molarity factors of the two solutions, and the equivalence factor for the substance given in the individual monograph.

COMPLEXOMETRIC TITRATIONS

Successful complexometric titrations depend on several factors. The equilibrium constant for formation of the titrant-analyte complex must be sufficiently large that, at the endpoint, very close to 100% of the analyte has been complexed. The final complex must be formed rapidly enough that the analysis time is practical. When the analytical reaction is not rapid, a residual titration may sometimes be successful.

In general, complexometric indicators are themselves complexing agents. The reaction between metal ion and indicator must be rapid and reversible. The equilibrium constant for formation of the metal-indicator complex should be large enough to produce a sharp color change but must be less than that for the metal-titrant complex. Indicator choice is also restricted by the pH range within which the complexation reaction must be carried out and by interference of other ions arising from the sample or the buffer. Interfering ions may often be masked or “screened” via addition of another complexing agent. (The masking technique is also applicable to redox titrations.)

OXIDATION-REDUCTION (REDOX) TITRATIONS

Determinations may often be carried out conveniently by the use of a reagent that brings about oxidation or reduction of the analyte. Many redox titration curves are not symmetric about the equivalence point, and thus graphical determination of the endpoint is not possible; but indicators are available for many determinations, and a redox reagent can often serve as its own indicator. As in any type of titration, the ideal indicator changes color at an endpoint that is as close as possible to the equivalence point. Accordingly, when the titrant serves as its own indicator, the difference between the endpoint and the equivalence point is determined only by the analyst’s ability to detect the color change. A common example is the use of permanganate ion as an oxidizing titrant since a slight excess can easily be detected by its pink color. Other titrants that may serve as their own indicators are iodine, cerium (IV) salts, and potassium dichromate. In most cases, however, the use of an appropriate redox indicator will yield a much sharper endpoint.

It may be necessary to adjust the oxidation state of the analyte prior to titration through use of an appropriate oxidizing or reducing agent; the excess reagent must then be removed, e.g., through precipitation. This is nearly always the practice in the determination of oxidizing agents since most volumetric solutions of reducing agents are slowly oxidized by atmospheric oxygen.
TITRATIONS IN NONAQUEOUS SOLVENTS

Acids and bases have long been defined as substances that furnish, when dissolved in water, hydrogen and hydroxyl ions, respectively. This definition, introduced by Arrhenius, fails to recognize the fact that properties characteristic of acids or bases may be developed also in other solvents. A more generalized definition is that of Brönsted, who defined an acid as a substance that furnishes protons, and a base as a substance that combines with protons. Even broader is the definition of Lewis, who defined an acid as any material that will accept an electron pair, a base as any material that will donate an electron pair, and neutralization as the formation of a coordination bond between an acid and a base.

The apparent strength of an acid or a base is determined by the extent of its reaction with a solvent. In water solution all strong acids appear equally strong because they react with the solvent to undergo almost complete conversion to oxonium ion and the acid anion (leveling effect). In a weakly protophilic solvent such as acetic acid the extent of formation of the acetate acidium ion shows that the order of decreasing strength for acids is perchloric, hydrobromic, sulfuric, hydrochloric, and nitric (differentiating effect).

Acetic acid reacts incompletely with water to form oxonium ion and is, therefore, a weak acid. In contrast, it dissolves in a base such as ethylenediamine, and reacts so completely with the solvent that it behaves as a strong acid. The same holds for perchloric acid.

This leveling effect is observed also for bases. In sulfuric acid almost all bases appear to be of the same strength. As the acid properties of the solvent decrease in the series sulfuric acid, acetic acid, phenol, water, pyridine, and butylamine, the bases become progressively weaker until all but the strongest have lost their basic properties. In order of decreasing strength, the strong bases are sodium 2-aminoethoxide, potassium methoxide, sodium methoxide, and lithium methoxide.

Many water-insoluble compounds acquire enhanced acidic or basic properties when dissolved in organic solvents. Thus the choice of the appropriate solvent permits the determination of a variety of such materials by nonaqueous titration. Furthermore, depending upon which part of a compound is the physiologically active moiety, it is often possible to titrate that part by proper selection of solvent and titrant. Pure compounds can be titrated directly, but it is often necessary to isolate the active ingredient in pharmaceutical preparations from interfering excipients and carriers.

The types of compounds that may be titrated as acids include acid halides, acid anhydrides, carboxylic acids, amino acids, enols such as barbiturates and xanthines, imides, phenols, pyroles, and sulfonamides. The types of compounds that may be titrated as bases include amines, nitrogen-containing heterocyclic compounds, oxazolines, quaternary ammonium compounds, alkali salts of organic acids, alkali salts of weak inorganic acids, and some salts of amines. Many salts of halogen acids may be titrated in acetic acid or acetic anhydride after the addition of mercuric acetate, which removes halide ion as the unionized mercuric halide complex and introduces the acetate ion.

For the titration of a basic compound, a volumetric solution of perchloric acid in glacial acetic acid is preferred, although perchloric acid in dioxane is used in special cases. The calomel-glass electrode system is useful in this case. In acetic acid solvent, this electrode system functions as predicted by theory.

For the titration of an acidic compound, two classes of titrant are available: the alkali metal alkoxides and the tetraalkylammonium hydroxides. A volumetric solution of sodium methoxide in a mixture of methanol and toluene is used frequently, although lithium methoxide in methanol-benzene solvent is used for those compounds yielding a gelatinous precipitate on titration with sodium methoxide.

The alkali error limits the use of the glass electrode as an indicating electrode in conjunction with alkali metal alkoxide titrants, particularly in basic solvents. Thus, the antimony-indicating electrode, though somewhat erratic, is used in such titrations. The use of quaternary ammonium hydroxide compounds, e.g., tetra-n-butylammonium hydroxide and trimethylhexadecylammonium hydroxide (in benzene-methanol or isopropyl alcohol), has two advantages over the other titrants in that (a) the tetraalkylammonium salt of the titrated acid is soluble in the titration medium, and (b) the convenient and well-behaved calomel-glass electrode pair may be used to conduct potentiometric titrations.

Because of interference by carbon dioxide, solvents for acidic compounds need to be protected from excessive exposure to the atmosphere by a suitable cover or by an inert atmosphere during the titration. Absorption of carbon dioxide may be determined by performing a blank titration. The blank should not exceed 0.01 mL of 0.1 N sodium methoxide VS per mL of solvent.

The endpoint may be determined visually by color change, or potentiometrically, as indicated in the individual monograph. If the calomel reference electrode is used, it is advantageous to replace the aqueous potassium chloride salt bridge with 0.1 N lithium perchlorate in glacial acetic acid for titrations in acidic solvents or potassium chloride in methanol for titrations in basic solvents.

Where these or other mixtures are specified in individual monographs, the calomel reference electrode is modified by first removing the aqueous potassium chloride solution and residual potassium chloride, if any, by rinsing with water, then eliminating residual water by rinsing with the required nonaqueous solvent, and finally filling the electrode with the designated nonaqueous mixture.

In nearly all cases, except those where silver ion might interfere, a silver-silver chloride reference electrode may be substituted for the calomel electrode. The silver-silver chloride electrode is more rugged, and its use helps to eliminate toxic mercury salts from the laboratory. Generally, a salt bridge may be used to circumvent interference by silver ion.

The more useful systems for titration in nonaqueous solvents are listed in Table 1.
Table 1. Systems for Nonaqueous Titrations

<table>
<thead>
<tr>
<th>Type of Solvent</th>
<th>Acidic (for titration of bases and their salts)</th>
<th>Relatively Neutral (for differential titration of bases)</th>
<th>Basic (for titration of acids)</th>
<th>Relatively Neutral (for differential titration of acids)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glacial Acetic Acid</td>
<td>Acetonitrile</td>
<td>Dimethylformamide</td>
<td>Acetone</td>
<td></td>
</tr>
<tr>
<td>Acetic Anhydride</td>
<td>Alcohols</td>
<td>n-Butylamine</td>
<td>Acetonitrile</td>
<td></td>
</tr>
<tr>
<td>Formic Acid</td>
<td>Chloroform</td>
<td>Pyridine</td>
<td>Methyl Ethyl Ketone</td>
<td></td>
</tr>
<tr>
<td>Propionic Acid</td>
<td>Toluene</td>
<td>Ethylenediamine</td>
<td>Methyl Isobutyl Ketone</td>
<td></td>
</tr>
<tr>
<td>Sulfuryl Chloride</td>
<td>Chlorobenzene</td>
<td>Morpholine</td>
<td>tert-Butyl Alcohol</td>
<td></td>
</tr>
<tr>
<td>Indicator</td>
<td>Crystal Violet</td>
<td>Thymol Blue</td>
<td>Azo Violet</td>
<td></td>
</tr>
<tr>
<td>Quinaldine Red</td>
<td>Methyl Orange</td>
<td>Thymolphthalein</td>
<td>Bromothylmol Blue</td>
<td></td>
</tr>
<tr>
<td>p-Naphtholbenzoine</td>
<td>p-Naphtholbenzoine</td>
<td>Azo Violet</td>
<td>p-Hydroxyazobenzene</td>
<td></td>
</tr>
<tr>
<td>Alphazurine 2-G</td>
<td>o-Nitroaniline</td>
<td>Thymol Blue</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malachite Green</td>
<td>p-Hydroxyazobenzene</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Electrodes</td>
<td>Glass–calomel</td>
<td>Antimony–calomel</td>
<td>Antimony–calomel</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Glass–silver–silver chloride</td>
<td>Antimony–glass</td>
<td>Glass–calomel</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mercury–mercuric acetate</td>
<td>Antimony–antimony2</td>
<td>Glass–platinum2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Platinum–calomel</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Glass–calomel</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 Relatively neutral solvents of low dielectric constant such as benzene, toluene, chloroform, or dioxane may be used in conjunction with any acidic or basic solvent in order to increase the sensitivity of the titration end-points.

2 In titrant.

INDICATOR AND POTENTIOMETRIC ENDPOINT DETECTION

The simplest and most convenient method by which the equivalence point, i.e., the point at which the stoichiometric analytical reaction is complete, may be determined is with the use of indicators. These chemical substances, usually colored, respond to changes in solution conditions before and after the equivalence point by exhibiting color changes that may be taken visually as the endpoint, a reliable estimate of the equivalence point.

A useful method of endpoint determination results from the use of electrochemical measurements. If an indicator electrode, sensitive to the concentration of the species undergoing titrimetric reaction, and a reference electrode, whose potential is insensitive to any dissolved species, are immersed in the titrate to form a galvanic cell, the potential difference between the electrodes may be sensed by a pH meter and used to follow the course of the reaction. Where such a series of measurements is plotted correctly (i.e., for an acid-base titration, pH versus mL of titrant added; for a precipitometric, complexometric, or oxidation-reduction titration, mV versus mL of titrant added), a sigmoid curve results with a rapidly changing portion (the “break”) in the vicinity of the equivalence point. The midpoint of this linear vertical portion or the inflection point may be taken as the endpoint. The equivalence point may also be determined mathematically without plotting a curve. However, it should be noted that in asymmetrical reactions, which are reactions in which the number of anions reacting is not the same as the number of cations reacting, the endpoint as defined by the inflection of the titration curve does not occur exactly at the stoichiometric equivalence point. Thus, potentiometric endpoint detection by this method is not suitable in the case of asymmetric reactions, examples of which are the precipitation reaction,

\[ 2\text{Ag}^+ + \text{CrO}_4^{2-} \]

and the oxidation-reduction reaction,

\[ 5\text{Fe}^{2+} + \text{MnO}_4^- \]

All acid-base reactions, however, are symmetrical. Thus, potentiometric endpoint detection may be employed in acid-base titrations and in other titrations involving symmetrical reversible reactions where an indicator is specified, unless otherwise directed in the individual monograph.

Two types of automatic electrometric titrators are available. The first is one that carries out titrant addition automatically and records the electrode potential differences during the course of titration as the expected sigmoid curve. In the second type, titrant addition is performed automatically until a preset potential or pH, representing the endpoint, is reached, at which point the titrant addition ceases.

Several acceptable electrode systems for potentiometric titrations are summarized in Table 2.


**BLANK CORRECTIONS**

As previously noted, the endpoint determined in a titrimetric assay is an estimate of the reaction equivalence point. The validity of this estimate depends upon, among other factors, the nature of the titrate constituents and the concentration of the substance being assayed. An appropriate **blank correction** is employed in titrimetric assays to enhance the reliability of the endpoint determination. Such a blank correction is usually obtained by means of a residual blank titration, wherein the required procedure is repeated in every detail except that the substance being assayed is omitted. In such instances, the actual volume of titrant consumed is 

<table>
<thead>
<tr>
<th>Titration</th>
<th>Indicating Electrode</th>
<th>Equation1</th>
<th>Reference Electrode</th>
<th>Applicability2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid-base</td>
<td>Glass</td>
<td>E = k + 0.0591 pH</td>
<td>Calomel or silver-silver chloride</td>
<td>Titration of acids and bases</td>
</tr>
<tr>
<td>Precipitometric (silver)</td>
<td>Silver</td>
<td>E = E' + 0.0591 log [Ag+]</td>
<td>Calomel (with potassium nitrate salt bridge)</td>
<td>Titration with or of silver involving halides or thiocyanates</td>
</tr>
<tr>
<td>Complexometric</td>
<td>Mercury-mercury(II)</td>
<td>E = E' + 0.0296(log k' – pM)</td>
<td>Calomel</td>
<td>Titration of various metals (M), e.g., Mg2+, Ca2+, Fe3+, with EDTA</td>
</tr>
<tr>
<td>Oxidation-reduction</td>
<td>Platinum</td>
<td>E = E' + (0.0591/n) x log [ox]/[red]</td>
<td>Calomel or silver-silver chloride</td>
<td>Titrations with arsenite, bromine, cerate, dichromate, exacyanoferrate(III), iodate, nitrite, permanganate, thiosulfate</td>
</tr>
</tbody>
</table>

1 Appropriate form of Nernst equation describing the indicating electrode system: k = glass electrode constant; k' = constant derived from Hg–Hg(II)–EDTA equilibrium; M = any metal undergoing EDTA titration; [ox] and [red] from the equation, ox + ne = red.

2 Listing is representative but not exhaustive.

**〈611〉 ALCOHOL DETERMINATION**

**PROCEDURES**

Method I—Distillation Method

Method I is to be used for the determination of alcohol, unless otherwise specified in the individual monograph. It is suitable for examining most fluidextracts and tinctures, provided the capacity of the distilling flask is sufficient (commonly two to four times the volume of the liquid to be heated) and the rate of distillation is such that clear distillates are produced. Cloudy distillates may be clarified by agitation with talc, or with calcium carbonate, and filtered, after which the temperature of the filtrate is adjusted and the alcohol content determined from the specific gravity. During all manipulations, take precautions to minimize the loss of alcohol by evaporation.

Treat liquids that froth to a troublesome extent during distillation by rendering them strongly acidic with phosphoric, sulfuric, or tannic acid, or treat with a slight excess of calcium chloride solution or with a small amount of paraffin or silicone oil before starting the distillation.

Prevent bumping during distillation by adding porous chips of insoluble material such as silicon carbide, or beads.

For liquids presumed to contain 30% of alcohol or less: By means of a pipet, transfer to a suitable distilling apparatus not less than 25 mL of the liquid in which the alcohol is to be determined, and note the temperature at which the volume was measured. Add an equal volume of water, distill, and collect a volume of distillate about 2 mL less than the volume taken of the original test liquid, adjust to the temperature at which the original test liquid was measured, add sufficient water to measure exactly the original volume of the test liquid, and mix. The distillate is clear or not more than slightly cloudy, and does not contain more than traces of volatile substances other than alcohol and water. Determine the specific gravity of the liquid at 25°, as directed under Specific Gravity (841), using this result to ascertain the percentage, by volume, of C₂H₅OH contained in the liquid examined by reference to the Alcoholometric Table in the section Reference Tables.

For liquids presumed to contain more than 30% of alcohol: Proceed as directed in the foregoing paragraph, except to do the following: dilute the specimen with about twice its volume of water, collect a volume of distillate about 2 mL less than twice the volume of the original test liquid, bring to the temperature at which the original liquid was measured, add sufficient water to measure exactly twice the original volume of the test liquid, mix, and determine its specific gravity. The proportion of C₂H₅OH, by volume, in this distillate, as ascertained from its specific gravity, equals one-half that in the liquid examined.

Special Treatment:

**Volatile acids and bases:** Render preparations containing volatile bases slightly acidic with diluted sulfuric acid before distilling. If volatile acids are present, render the preparation slightly alkaline with sodium hydroxide T.S.
**Glycerin:** To liquids that contain glycerin add sufficient water so that the residue, after distillation, contains not less than 50% of water.

**Iodine:** Treat all solutions containing free iodine with powdered zinc before the distillation, or decolorize with just sufficient sodium thiosulfate solution (1 in 10), followed by a few drops of sodium hydroxide TS.

**Other volatile substances:** Spirits, elixirs, tinctures, and similar preparations that contain appreciable proportions of volatile materials other than alcohol and water, such as volatile oils, chloroform, ether, camphor, etc., require special treatment, as follows:

For liquids presumed to contain 50% of alcohol or less: Mix 25 mL of the specimen under examination, accurately measured, with about an equal volume of water in a separator. Saturate this mixture with sodium chloride, then add 25 mL of solvent hexane, and shake the mixture to extract the interfering volatile ingredients. Draw off the separated, lower layer into a second separator, and repeat the extraction twice with two further 25-mL portions of solvent hexane. Extract the combined solvent hexane solutions with three 10-mL portions of a saturated solution of sodium chloride. Combine the saline solutions, and distill in the usual manner, collecting a volume of distillate having a simple ratio to the volume of the original specimen.

For liquids presumed to contain more than 50% of alcohol: Adjust the specimen under examination to a concentration of approximately 25% of alcohol by diluting it with water, then proceed as directed in For liquids presumed to contain 50% of alcohol or less, beginning with “Saturate this mixture with sodium chloride.”

In preparing **Collodion or Flexible Collodion** for distillation, use water in place of the saturated solution of sodium chloride directed above.

If volatile oils are present in small proportions only, and a cloudy distillate is obtained, the solvent hexane treatment not having been employed, the distillate may be clarified and rendered suitable for the specific gravity determination by shaking it with about one-fifth its volume of solvent hexane, or by filtering it through a thin layer of t alc.

**Method II—Gas Chromatographic Method**

Use **Method IIA** when **Method II** is specified in the individual monograph. For a discussion of the principles upon which it is based, see **Gas Chromatography** under **Chromatography** (621).

**• METHOD IIA**

**Apparatus:** Under typical conditions, use a gas chromatograph equipped with a flame-ionization detector and a 4-mm × 1.8-m glass column packed with 100- to 120-mesh chromatographic column packing support S3, using nitrogen or helium as the carrier. Prior to use, condition the column overnight at 235° with a slow flow of carrier gas. The column temperature is maintained at 120°, and the injection port and detector temperatures are maintained at 210°. Adjust the carrier flow and temperature so that acetonitrile, the internal standard, elutes in 5 to 10 minutes.

**Solutions**

**Test stock preparation:** Dilute the specimen under examination stepwise with water to obtain a solution containing approximately 2% (v/v) of alcohol.

**Test preparation:** Pipet 5 mL each of the **Test stock preparation** and the USP Alcohol Determination—Acetonitrile RS [**NOTE**—Alternatively, a 2% aqueous solution of acetonitrile of suitable quality may be used as the internal standard solution] into a 50-mL volumetric flask, dilute with water to volume, and mix.

**Standard preparation:** Pipet 5 mL each of the USP Alcohol Determination—Alcohol RS and the USP Alcohol Determination—Acetonitrile RS. [**NOTE**—Alternatively, a 2% aqueous solution of acetonitrile of suitable quality may be used as the internal standard solution] into a 50-mL volumetric flask, dilute with water to volume, and mix.

**Procedure:** Inject about 5 µL each of the **Test preparation** and the **Standard preparation**, in duplicate, into the gas chromatograph, record the chromatograms, and determine the peak response ratios. Calculate the percentage of alcohol (v/v) in the specimen under test according to the formula:

\[
\text{Result} = \frac{CD}{R_\text{usp}/R_\text{rs}}
\]

- \( C \) = labeled concentration of USP Alcohol Determination—Alcohol RS
- \( D \) = dilution factor (the ratio of the volume of the **Test stock preparation** to the volume of the specimen taken)
- \( R_\text{usp} \) = peak response ratio obtained from the **Test preparation**
- \( R_\text{rs} \) = peak response ratio obtained from the **Standard preparation**

**System suitability test:** In a suitable chromatogram, the resolution factor, \( R \), is not less than 2; the tailing factor of the alcohol peak is not greater than 2.0; and six replicate injections of the **Standard preparation** show a relative standard deviation of not more than 2.0% in the ratio of the peak of alcohol to the peak of the internal standard.

**• METHOD III**

**Apparatus:** The gas chromatograph is equipped with a split injection port with a split ratio of 5:1, a flame-ionization detector, and a 0.53-mm × 30-m capillary column coated with a 3.0-µm film of phase G43. Helium is used as the carrier gas at a linear velocity of 34.0 cm per second. The chromatograph is programmed to maintain the column temperature at 50° for 5 minutes, then to increase the temperature at a rate of 10° per minute to 200°, and maintain at this temperature for 4 minutes. The injection port temperature is maintained at 210° and the detector temperature at 280°.

**Solutions**

**Test stock preparation:** Dilute the specimen under examination stepwise with water to obtain a solution containing approximately 2% (v/v) of alcohol.

**Test preparation:** Pipet 5 mL each of the **Test stock preparation** and the USP Alcohol Determination—Acetonitrile RS [**NOTE**—Alternatively, a 2% aqueous solution of acetonitrile of suitable quality may be used as the internal standard solution] into a 25-mL volumetric flask, dilute with water to volume, and mix.
Standard preparation: Pipet 5 mL each of the USP Alcohol Determination—Alcohol RS and the USP Alcohol Determination—Acetonitrile RS. [Note—Alternatively, a 2% aqueous solution of acetonitrile of suitable quality may be used as the internal standard solution] into a 25-mL volumetric flask, dilute with water to volume, and mix.

Procedure: Inject about 0.2 to 0.5 µL each of the Test preparation and the Standard preparation, in duplicate, into the gas chromatograph, record the chromatograms, and determine the peak response ratios. Calculate the percentage of alcohol (v/v) in the specimen under test according to the formula:

\[
\text{Result} = CD\left(\frac{R_U}{R_S}\right)
\]

- \(C\) = labeled concentration of USP Alcohol Determination—Alcohol RS
- \(D\) = dilution factor (the ratio of the volume of the Test stock preparation to the volume of the specimen taken)
- \(R_U\) = peak response ratio obtained from the Test preparation
- \(R_S\) = peak response ratio obtained from the Standard preparation

System suitability test: In a suitable chromatogram, the resolution factor, \(R\), between alcohol and the internal standard is not less than 4; the tailing factor of the alcohol peak is not greater than 2.0; and six replicate injections of the Standard preparation show a relative standard deviation of not more than 4.0% in the ratio of the peak of alcohol to the peak of the internal standard.

ADDITIONAL REQUIREMENTS
- USP Reference Standards (11)
  - USP Alcohol Determination—Acetonitrile RS
  - USP Alcohol Determination—Alcohol RS

(621) CHROMATOGRAPHY

INTRODUCTION

Chromatographic separation techniques are multistage separation methods in which the components of a sample are distributed between two phases, of which one is stationary and the other is mobile. The stationary phase may be a solid or a liquid supported on a solid or a gel. The stationary phase may be packed in a column, spread as a layer, distributed as a film, or applied by other techniques. The mobile phase may be in a gaseous or liquid form, or a supercritical fluid. The separation may be based on adsorption, mass distribution (partition), or ion exchange; or it may be based on differences among the physicochemical properties of the molecules, such as size, mass, and volume. This chapter contains general procedures, definitions, and calculations of common parameters and describes general requirements for system suitability. The types of chromatography useful in qualitative and quantitative analyses employed in USP procedures are column, gas (GC), paper, thin-layer (TLC) [including high-performance thin-layer chromatography (HPTLC)], and pressurized liquid chromatography [commonly called high-pressure or high-performance liquid chromatography (HPLC)].

GENERAL PROCEDURES

This section describes the basic procedures used when a chromatographic method is described in a monograph. These procedures are followed unless otherwise indicated in the individual monograph.

Paper Chromatography

STATIONARY PHASE

The stationary phase is a sheet of paper of suitable texture and thickness. Development may be ascending, in which the solvent is carried up the paper by capillary forces, or descending, in which the solvent flow is also assisted by gravitational force. The orientation of paper grain, with respect to solvent flow, is to be kept constant in a series of chromatograms. The machine direction is usually designated by the manufacturer.

APPARATUS

The essential equipment for paper chromatography consists of a vapor-tight chamber with inlets for the addition of solvent and a rack of corrosion-resistant material about 5 cm shorter than the inside height of the chamber. The rack serves as a support for solvent troughs and antisiphon rods that, in turn, hold up the chromatographic sheets. The bottom of the chamber is covered with the prescribed solvent system or mobile phase. Saturation of the chamber with solvent vapor is facilitated by lining the inside walls with paper wetted with the prescribed solvent system.

SPOTTING

The substance or substances analyzed are dissolved in a suitable solvent. Convenient volumes delivered from suitable micropipettes of the resulting solution, normally containing 1–20 µg of the compound, are placed in 6- to 10-mm spots NLT 3 cm apart.
DESCENDING PAPER CHROMATOGRAPHY PROCEDURE

1. A spotted chromatographic sheet is suspended in the apparatus, using the antisiphon rod to hold the upper end of the sheet in the solvent trough. [NOTE—Ensure that the portion of the sheet hanging below the rods is freely suspended in the chamber without touching the rack, the chamber walls, or the fluid in the chamber.]
2. The chamber is sealed to allow equilibration (saturation) of the chamber and the paper with the solvent vapor. Any excess pressure is released as necessary.
3. After equilibration of the chamber, the prepared mobile phase is introduced into the trough through the inlet.
4. The inlet is closed, and the mobile solvent phase is allowed to travel the desired distance down the paper.
5. The sheet is removed from the chamber.
6. The location of the solvent front is quickly marked, and the sheet is dried.
7. The chromatogram is observed and measured directly or after suitable development to reveal the location of the spots of the isolated drug or drugs.

ASCENDING PAPER CHROMATOGRAPHY PROCEDURE

1. The mobile phase is added to the bottom of the chamber.
2. The chamber is sealed to allow equilibration (saturation) of the chamber and the paper with the solvent vapor. Any excess pressure is released as necessary.
3. The lower edge of the stationary phase is dipped into the mobile phase to permit the mobile phase to rise on the chromatographic sheet by capillary action.
4. When the solvent front has reached the desired height, the chamber is opened, the sheet is removed, the location of the solvent front is quickly marked, and the sheet is dried.
5. The chromatogram is observed and measured directly or after suitable development to reveal the location of the spots of the isolated drug or drugs.

Thin-Layer Chromatography

STATIONARY PHASE

The stationary phase is a relatively thin, uniform layer of dry, finely powdered material applied to a glass, plastic, or metal sheet or plate (typically called the plate). The stationary phase of TLC plates has an average particle size of 10–15 µm, and that of HPTLC plates has an average particle size of 5 µm. Commercial plates with a preadsorbent zone can be used if they are specified in a monograph. The sample applied to the preadsorbent region develops into sharp, narrow bands at the preadsorbent–sorbent interface. The separations achieved may be based on adsorption, partition, or a combination of both effects, depending on the particular type of stationary phase.

APPARATUS

A chromatographic chamber made of inert, transparent material and having the following specifications is used: a flat-bottom or twin trough, a tightly fitted lid, and a size suitable for the plates. The chamber is lined on at least one wall with filter paper. Sufficient mobile phase or developing solvent is added to the chamber so that, after impregnation of the filter paper, a depth appropriate to the dimensions of the plate used is available. The chromatographic chamber is closed and allowed to equilibrate. [NOTE—Unless otherwise indicated, the chromatographic separations are performed in a saturated chamber.]

DETECTION/VISUALIZATION

An ultraviolet (UV) light source suitable for observations under short- (254 nm) and long- (365 nm) wavelength UV light and a variety of other spray reagents, used to make spots visible, is often used.

SPOTTING

Solutions are spotted on the surface of the stationary phase (plate) at the prescribed volume in sufficiently small portions to obtain circular spots of 2–5 mm in diameter (1–2 mm on HPTLC plates) or bands of 10–20 mm × 1–2 mm (5–10 mm × 0.5–1 mm on HPTLC plates) at an appropriate distance from the lower edge and sides of the plate. [NOTE—During development, the application position must be at least 5 mm (TLC) or 3 mm (HPTLC) above the level of the mobile phase.] The solutions are applied on a line parallel to the lower edge of the plate with an interval of at least 10 mm (5 mm on HPTLC plates) between the centers of spots or 4 mm (2 mm on HPTLC plates) between the edges of bands, then allowed to dry.

PROCEDURE

1. Place the plate in the chamber, ensuring that the spots or bands are above the surface of the mobile phase.
2. Close the chamber.
3. Allow the mobile phase to ascend the plate until the solvent front has traveled three-quarters of the length of the plate, or the distance prescribed in the monograph.
4. Remove the plate, mark the solvent front with a pencil, and allow to dry.
5. Visualize the chromatograms as prescribed.
6. Determine the chromatographic Retardation factor \( (R_F) \) values for the principal spots or zones.

7. Presumptive identification can be made by observation of spots or zones of identical \( R_F \) value and about equal magnitude obtained, respectively, with an unknown and a standard chromatographed on the same plate. A visual comparison of the size or intensity of the spots or zones may serve for semiquantitative estimation. Quantitative measurements are possible by means of densitometry (absorbance or fluorescence measurements).

**Column Chromatography**

**SOLID SUPPORT**

Purified siliceous earth is used for normal-phase separation. Silanized chromatographic siliceous earth is used for reverse-phase partition chromatography.

**STATIONARY PHASE**

The solid support is modified by the addition of a stationary phase specified in the individual monograph. If a mixture of liquids is used as the stationary phase, mix the liquids before the introduction of the solid support.

**MOBILE PHASE**

The mobile phase is specified in the individual monograph. If the stationary phase is an aqueous solution, equilibrate with water. If the stationary phase is a polar organic fluid, equilibrate with that fluid.

**APPARATUS**

Unless otherwise specified in the individual monograph, the chromatographic tube is about 22 mm in its inside diameter and 200–300 mm long. Attached to it is a delivery tube, without stopcock, about 4 mm in its inside diameter and about 50 mm long.

**Apparatus preparation:** Pack a pledget of fine glass wool in the base of the tube. Combine the specified volume of stationary phase and the specified amount of solid support to produce a homogeneous, fluffy mixture. Transfer this mixture to the chromatographic tube, and tamp using gentle pressure to obtain a uniform mass. If the specified amount of solid support is >3 g, transfer the mixture to the column in portions of approximately 2 g, and tamp each portion. If the assay or test requires a multisegment column with a different stationary phase specified for each segment, tamp after the addition of each segment, and add each succeeding segment directly to the previous one. Pack a pledget of fine glass wool above the completed column packing. [Note—The mobile phase should flow through a properly packed column as a moderate stream or, if reverse-phase chromatography is applied, as a slow trickle.]

If a solution of the analyte is incorporated into the stationary phase, complete the quantitative transfer to the chromatographic tube by scrubbing the beaker used for the preparation of the test mixture with a mixture of about 1 g of solid support and several drops of the solvent used to prepare the sample solution before adding the final portion of glass wool.

**PROCEDURE**

1. Transfer the mobile phase to the column space above the column packing, and allow it to flow through the column under the influence of gravity.
2. Rinse the tip of the chromatographic column with about 1 mL of mobile phase before each change in composition of mobile phase and after completion of the elution.
3. If the analyte is introduced into the column as a solution in the mobile phase, allow it to pass completely into the column packing, then add the mobile phase in several small portions, allowing each to drain completely, before adding the bulk of the mobile phase.
4. Where the procedure indicates the use of multiple chromatographic columns mounted in series and the addition of mobile phase in divided portions is specified, allow each portion to drain completely through each column, and rinse the tip of each with mobile phase before the addition of each succeeding portion.

**Gas Chromatography**

**LIQUID STATIONARY PHASE**

This type of phase is available in packed or capillary columns.

**PACKED COLUMN GAS CHROMATOGRAPHY**

The liquid stationary phase is deposited on a finely divided, inert solid support, such as diatomaceous earth, porous polymer, or graphitized carbon, which is packed into a column that is typically 2–4 mm in internal diameter and 1–3 m in length.

**CAPILLARY COLUMN GAS CHROMATOGRAPHY**

In capillary columns, which contain no packed solid support, the liquid stationary phase is deposited on the inner surface of the column and may be chemically bonded to it.
SOLID STATIONARY PHASE

This type of phase is available only in packed columns. In these columns the solid phase is an active adsorbent, such as alumina, silica, or carbon, packed into a column. Polyaromatic porous resins, which are sometimes used in packed columns, are not coated with a liquid phase. [NOTE—Packed and capillary columns must be conditioned before use until the baseline and other characteristics are stable. The column or packing material supplier provides instructions for the recommended conditioning procedure.]

APPARATUS

A gas chromatograph consists of a carrier gas source, injection port, column, detector, and recording device. The injection port, column, and detector are temperature controlled and may be varied as part of the analysis. The typical carrier gas is helium, nitrogen, or hydrogen, depending on the column and detector in use. The type of detector used depends on the nature of the compounds analyzed and is specified in the individual monograph. Detector output is recorded as a function of time, and the instrument response, measured as peak area or peak height, is a function of the amount present.

TEMPERATURE PROGRAM

The length and quality of a GC separation can be controlled by altering the temperature of the chromatographic column. When a temperature program is necessary, the individual monograph indicates the conditions in table format. The table indicates the initial temperature, rate of temperature change (ramp), final temperature, and hold time at the final temperature.

PROCEDURE

1. Equilibrate the column, injector, and detector with flowing carrier gas until a constant signal is received.
2. Inject a sample through the injector septum, or use an autosampler.
3. Begin the temperature program.
4. Record the chromatogram.
5. Analyze as indicated in the monograph.

Liquid Chromatography

LC, as used in the compendia, is synonymous with HPLC (both high-pressure and high-performance). LC is a separation technique based on a solid stationary phase and a liquid mobile phase.

STATIONARY PHASE

Separations are achieved by partition, adsorption, or ion-exchange processes, depending on the type of stationary phase used. The most commonly used stationary phases are modified silica or polymeric beads. The beads are modified by the addition of long-chain hydrocarbons. The specific type of packing needed to complete an analysis is indicated by the “L” designation in the individual monograph (see also Chromatographic Columns). The size of the beads is often described in the monograph as well. Changes in the packing type and size are covered in System Suitability in this chapter.

CHROMATOGRAPHIC COLUMN

The term “column” includes stainless steel, lined stainless steel, and polymeric columns, packed with a stationary phase. The length and inner diameter of the column affects the separation, and therefore typical column dimensions are included in the individual monograph. Changes to column dimensions are discussed in System Suitability. Compendial monographs do not include the name of appropriate columns; this omission avoids the appearance of endorsement of a vendor’s product and natural changes in the marketplace. See Chromatographic Columns for more information.

In LC procedures, a guard column may be used with the following requirements, unless otherwise indicated in the individual monograph: (a) the length of the guard column must be NMT 15% of the length of the analytical column, (b) the inner diameter must be the same or smaller than that of the analytical column, and (c) the packing material should be the same as the analytical column (e.g., silica) and contain the same bonded phase (e.g., C18). In any case, all system suitability requirements specified in the official procedure must be met with the guard column installed.

MOBILE PHASE

The mobile phase is a solvent or a mixture of solvents, as defined in the individual monograph.

APPARATUS

A liquid chromatograph consists of a reservoir containing the mobile phase, a pump to force the mobile phase through the system at high pressure, an injector to introduce the sample into the mobile phase, a chromatographic column, a detector, and a data collection device.
**GRADIENT ELUTION**

The technique of continuously changing the solvent composition during the chromatographic run is called gradient elution or solvent programming. The gradient elution profile is presented in the individual monograph as a gradient table, which lists the time and proportional composition of the mobile phase at the stated time.

**PROCEDURE**

1. Equilibrate the column and detector with mobile phase at the specified flow rate until a constant signal is received.
2. Inject a sample through the injector, or use an autosampler.
3. Begin the gradient program.
4. Record the chromatogram.
5. Analyze as directed in the monograph.

**CHROMATOGRAPHIC COLUMNS**

A complete list of packings (L), phases (G), and supports (S) used in USP–NF tests and assays is located in USP–NF, Reagents, Indicators, and Solutions—Chromatographic Columns. This list is intended to be a convenient reference for the chromatographer in identifying the pertinent chromatographic column specified in the individual monograph.

**DEFINITIONS AND INTERPRETATION OF CHROMATOGRAMS**

Chromatogram: A graphical representation of the detector response, concentration of analyte in the effluent, or other quantity used as a measure of effluent concentration versus effluent volume or time. In planar chromatography, chromatogram may refer to the paper or layer with the separated zones.

Figure 1 represents a typical chromatographic separation of two substances, 1 and 2. \( t_R1 \) and \( t_R2 \) are the respective retention times; \( h \) is the height, \( h/2 \) is the half-height, and \( W_{h/2} \) is the width at half-height, for peak 1. \( W_1 \) and \( W_2 \) are the respective widths of peaks 1 and 2 at the baseline. Air peaks are a feature of gas chromatograms and correspond to the solvent front in LC. The retention time of these air peaks, or unretained components, is designated as \( t_M \).

---

**Dwell volume (D):** Also known as “gradient delay volume”, is the volume between the point at which the eluents meet and the top of the column.

**Hold-up time (\( t_M \)):** The time required for elution of an unretained component (see Figure 1, shown as an air or unretained solvent peak, with the baseline scale in minutes).

**Hold-up volume (\( V_M \)):** The volume of mobile phase required for elution of an unretained component. It may be calculated from the hold-up time and the flow rate, \( F \), in mL/min:

\[
V_M = t_M \times F
\]

In size-exclusion chromatography, the symbol \( V_O \) is used.

**Number of theoretical plates (N):** A measure of column efficiency. For Gaussian peaks, it is calculated by:

\[
N = 16\left(\frac{t_M}{W}\right)^2
\]

1 The parameters \( k, N, r, \) and \( t_e \) were developed for isothermal GC separations and isocratic HPLC separations. Because these terms are thermodynamic parameters, they are only valid for separations made at a constant temperature, mobile phase composition, and flow rate. However, for separations made with a temperature program or solvent gradient, these parameters may be used simply as comparative means to ensure that adequate chromatographic conditions exist to perform the methods as intended in the monographs.
where \( t_R \) is the retention time of the substance, and \( W \) is the peak width at its base, obtained by extrapolating the relatively straight sides of the peak to the baseline. The value of \( N \) depends upon the substance being chromatographed as well as the operating conditions, such as the flow rate and temperature of the mobile phase or carrier gas, the quality of the packing, the uniformity of the packing within the column, and, for capillary columns, the thickness of the stationary phase film and the internal diameter and length of the column.

Where electronic integrators are used, it may be convenient to determine the number of theoretical plates, by the equation:

\[
N = 5.54 \left( \frac{t_R}{W_{1/2}} \right)^2
\]

where \( W_{1/2} \) is the peak width at half-height. However, in the event of dispute, only equations based on peak width at baseline are to be used.

**Peak**: The portion of the chromatographic recording of the detector response when a single component is eluted from the column. If separation is incomplete, two or more components may be eluted as one unresolved peak.

**Peak-to-valley ratio (p/v)**: \( p/v \) may be employed as a system suitability criterion in a test for related substances when baseline separation between two peaks is not achieved. Figure 2 represents a partial separation of two substances, where \( H_p \) is the height above the extrapolated baseline of the minor peak and \( H_v \) is the height above the extrapolated baseline at the lowest point of the curve separating the minor and major peaks:

\[
p/v = H_p/H_v
\]

Figure 2. Peak-to-valley ratio determination.

**Relative retardation \( (R_{rel}) \)**: The ratio of the distance traveled by the analyte to the distance simultaneously traveled by a reference compound (see Figure 3) and is used in planar chromatography.

\[
R_{rel} = b/c
\]

Figure 3. Typical planar chromatography.
Relative retention ($r$): The ratio of the adjusted retention time of a component relative to that of another used as a reference, obtained under identical conditions:

$$ r = \frac{(t_R^2 - t_M)}{(t_R^1 - t_M)} $$

where $t_R^2$ is the retention time measured from the point of injection of the compound of interest; $t_R^1$ is the retention time measured from the point of injection of the compound used as reference; and $t_M$ is the retention time of a nonretained marker defined in the procedure, all determined under identical experimental conditions on the same column.

Relative retention time (RRT): Also known as the “unadjusted relative retention”. Comparisons in USP–NF are normally made in terms of unadjusted relative retention, unless otherwise indicated.

$$ RRT = \frac{t_R^2}{t_R^1} $$

The symbol $r_G$ is also used to designate unadjusted relative retention values.

Relative standard deviation in percentage (%RSD):

$$ \%RSD = 100 \sqrt{\frac{1}{N-1} \sum_{i=1}^{N} \left( \frac{x_i - \bar{x}}{N-1} \right)^2} $$

Resolution ($R_s$): The resolution is the separation of two components in a mixture, calculated by:

$$ R_s = 2 \times \frac{(t_R^2 - t_R^1)}{(W_1 + W_2)} $$

where $t_R^2$ and $t_R^1$ are the retention times of the two components; and $W_2$ and $W_1$ are the corresponding widths at the bases of the peaks obtained by extrapolating the relatively straight sides of the peaks to the baseline.

Where electronic integrators are used, it may be convenient to determine the resolution, by the equation:

$$ R_s = 1.18 \times \frac{(t_R^2 - t_R^1)}{(W_{1,h/2} + W_{2,h/2})} $$

Retardation factor ($R_f$): The ratio of the distance traveled by the center of the spot to the distance simultaneously traveled by the mobile phase and is used in planar chromatography. Using the symbols in Figure 3:

$$ R_f = \frac{b}{a} $$

Retention factor ($k$): Also known as the “capacity factor ($k'$)”. Defined as:

$$ k = \frac{\text{amount of substance in stationary phase}}{\text{amount of substance in mobile phase}} $$

or

$$ k = \frac{\text{time spent by substance in stationary phase}}{\text{time spent by substance in mobile phase}} $$

The $k$ of a component may be determined from the chromatogram:

$$ k = \frac{(t_R - t_M)}{t_M} $$

Retention time ($t_R$): In LC and GC, the retention time, $t_R$, is defined as the time elapsed between the injection of the sample and the appearance of the maximum peak response of the eluted sample zone. $t_R$ may be used as a parameter for identification. Chromatographic retention times are characteristic of the compounds they represent but are not unique. The coincidence of retention times of a sample and a reference substance can be used as a partial criterion in construction of an identity profile, but may not be sufficient on its own to establish identity. Absolute retention times of a given compound may vary from one chromatogram to the next.

Retention volume ($V_R$): The volume of mobile phase required for elution of a component. It may be calculated from the retention time and the flow rate in mL/min:

$$ V_R = t_R \times F $$

Separation factor ($\alpha$): The relative retention calculated for two adjacent peaks (by convention, the value of the separation factor is always $>1$):

$$ \alpha = \frac{k_2}{k_1} $$
Symmetry factor \((A_s)\): Also known as the “tailing factor”, of a peak (see Figure 4) is calculated by:

\[
A_s = \frac{W_{0.05}}{2f}
\]

where \(W_{0.05}\) is the width of the peak at 5% height and \(f\) is the distance from the peak maximum to the leading edge of the peak, the distance being measured at a point 5% of the peak height from the baseline.

Tailing factor \((T)\): See Symmetry factor.

SYSTEM SUITABILITY

System suitability tests are an integral part of GC and LC methods. These tests are used to verify that the chromatographic system is adequate for the intended analysis.

The tests are based on the concept that the equipment, electronics, analytical operations, and samples analyzed constitute an integral system that can be evaluated as such.

Factors that may affect chromatographic behavior include the following:

- Composition, ionic strength, temperature, and apparent pH of the mobile phase
- Flow rate, column dimensions, column temperature, and pressure
- Stationary phase characteristics, including type of chromatographic support (particle-based or monolithic), particle or macropore size, porosity, and specific surface area
- Reverse-phase and other surface modification of the stationary phases, the extent of chemical modification (as expressed by end-capping, carbon loading, and others)

\(R_s\) is a function of the number of theoretical plates, \(N\) (also referred to as efficiency), \(\alpha\), and \(k\). [NOTE—All terms and symbols are defined in Definitions and Interpretation of Chromatograms.] For a given stationary phase and mobile phase, \(N\) may be specified to ensure that closely eluting compounds are resolved from each other, to establish the general resolving power of the system, and to ensure that internal standards are resolved from the drug. This is a less reliable means to ensure resolution, as opposed to direct measurement. Column efficiency is, in part, a reflection of peak sharpness, which is important for the detection of trace components.

Replicate injections of a standard preparation or other standard solutions are compared to ascertain whether requirements for precision are met. Unless otherwise specified in the individual monograph, data from five replicate injections of the analyte are used to calculate the relative standard deviation (RSD), if the requirement is \(\leq 2.0\%\); data from six replicate injections are used if the RSD requirement is >2.0%.

For the assay in a drug substance monograph, where the value is 100% for the pure substance, and no maximum RSD is stated, the maximum permitted %RSD is calculated for a series of injections of the reference solution:

\[
\%\text{RSD} = KB\sqrt{n}/t_{0.05, n-1}
\]

where \(K\) is a constant (0.349), obtained from the expression \(K = (0.6/\sqrt{2}) \times (t_{0.05, n}/\sqrt{6})\), in which 0.6/\sqrt{2} represents the required %RSD after six injections for \(B = 1.0\); \(B\) is the upper limit given in the definition of the individual monograph – 100%; \(n\) is the number of replicate injections of the reference solution \((3 \leq n \leq 6)\); and \(t_{0.05, n-1}\) is the Student’s \(t\) at the 90% probability level (double sided) with \(n-1\) degrees of freedom.

Unless otherwise prescribed, the maximum permitted RSD does not exceed the appropriate value given in Table 1 of repeatability requirements. This requirement does not apply to tests for related substances.

<table>
<thead>
<tr>
<th>Number of Individual Injections</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
</table>

\(2\) It is also a common practice to measure the “Asymmetry factor” as the ratio of the distance between the vertical line connecting the peak apex with the interpolated baseline and the peak front, and the distance between that line and the peak back measured at 10% of the peak height (see Figure 4), which would be \((W_{0.10} - f_{0.10})/f_{0.10}\). However, for the purposes of USP, only the formula \((A_s)\) as presented here is valid.
Table 1. RSD Requirements (continued)

<table>
<thead>
<tr>
<th>Number of Individual Injections</th>
<th>Maximum Permitted RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>B (%)</td>
<td></td>
</tr>
<tr>
<td>2.0</td>
<td>0.41 0.59 0.73 0.85</td>
</tr>
<tr>
<td>2.5</td>
<td>0.52 0.74 0.92 1.06</td>
</tr>
<tr>
<td>3.0</td>
<td>0.62 0.89 1.10 1.27</td>
</tr>
</tbody>
</table>

A, a measure of peak symmetry, is unity for perfectly symmetrical peaks; its value increases as tailing becomes more pronounced (see Figure 4). In some cases, values less than unity may be observed. As peak symmetry moves away from values of 1, integration, and hence precision, become less reliable.

The signal-to-noise (S/N) ratio is a useful system suitability parameter. The S/N ratio is calculated as follows:

\[
S/N \text{ ratio} = \frac{2H}{h}
\]

where \(H\) is the height of the peak measured from the peak apex to a baseline extrapolated over a distance \(≥\) 5 times the peak width at its half-height; and \(h\) is the difference between the largest and smallest noise values observed over a distance \(≥\) 5 times the width at the half-height of the peak and, if possible, situated equally around the peak of interest (see Figure 5).

Figure 5. Noise and chromatographic peak, components of the S/N ratio.

These system suitability tests are performed by collecting data from replicate injections of standard or other solutions as specified in the individual monograph.

The specification of definitive parameters in a monograph does not preclude the use of other suitable operating conditions. Adjustments to the specified chromatographic system may be necessary in order to meet system suitability requirements. Adjustments to chromatographic systems performed in order to comply with system suitability requirements are not to be made in order to compensate for column failure or system malfunction. Adjustments are permitted only when suitable standards (including Reference Standards) are available for all compounds used in the suitability test, and the adjustments or column change yields a chromatogram that meets all the system suitability requirements specified in the official procedure.

If adjustments of operating conditions are necessary in order to meet system suitability requirements, each of the items in the following list is the maximum variation that can be considered, unless otherwise directed in the monograph; these changes may require additional verification data. To verify the suitability of the method under the new conditions, assess the relevant analytical performance characteristics potentially affected by the change. Multiple adjustments can have a cumulative effect on the performance of the system and are to be considered carefully before implementation. In some circumstances, it may be desirable to use an HPLC column with different dimensions to those prescribed in the official procedure (different length, internal diameter, and/or particle size). In either case, changes in the chemical characteristics (“L” designation) of the stationary phase will be considered a modification to the method and will require full validation. Adjustments to the composition of the mobile phase in gradient elution may cause changes in selectivity and are not recommended. If adjustments are necessary, a change in column packing (maintaining the same chemistry), the duration of an initial isocratic hold (when prescribed), and/or the dwell volume are allowed. Additional allowances for gradient adjustment are noted in the text below.

- **pH of mobile phase (HPLC):** The pH of the aqueous buffer used in the preparation of the mobile phase can be adjusted to within ±0.2 units of the value or range specified. Applies to both gradient and isocratic separations.
- **Concentration of salts in buffer (HPLC):** The concentration of the salts used in the preparation of the aqueous buffer employed in the mobile phase can be adjusted to within ±10% if the permitted pH variation (see above) is met. Applies to both gradient and isocratic separations.
- **Ratio of components in mobile phase (HPLC):** The following adjustment limits apply to minor components of the mobile phase (specified as ≤50%). The amounts of these components can be adjusted by ±30% relative. However, the change in any component cannot exceed ±10% absolute (i.e., in relation to the total mobile phase). Adjustment can be made to one minor component in a ternary mixture. Examples of adjustments for binary and ternary mixtures are given below.
Binary mixtures

**SPECIFIED RATIO OF 50:50:** 30% of 50 is 15% absolute, but this exceeds the maximum permitted change of ±10% absolute in either component. Therefore, the mobile phase ratio may be adjusted only within the range of 40:60–60:40.

**SPECIFIED RATIO OF 2:98:** 30% of 2 is 0.6% absolute. Therefore, the maximum allowed adjustment is within the range of 1.4:98.6–2.6:97.4.

Ternary mixtures

**SPECIFIED RATIO OF 60:35:5:** For the second component, 30% of 35 is 10.5% absolute, which exceeds the maximum permitted change of ±10% absolute in any component. Therefore, the second component may be adjusted only within the range of 25%–45% absolute. For the third component, 30% of 5 is 1.5% absolute. In all cases, a sufficient quantity of the first component is used to give a total of 100%. Therefore, mixture ranges of 40:60–60:40.

Wavelength of UV-visible detector (HPLC): Deviations from the wavelengths specified in the procedure are not permitted. The procedure specified by the detector manufacturer, or another validated procedure, is used to verify that error in the detector wavelength is, at most, ±3 nm.

Stationary phase

**COLUMN LENGTH (GC):** Can be adjusted by as much as ±70%.

**COLUMN LENGTH (HPLC):** See Particle size (HPLC) below.

**COLUMN INNER DIAMETER (HPLC):** Can be adjusted if the linear velocity is kept constant. See Flow rate (HPLC).

**COLUMN INNER DIAMETER (GC):** Can be adjusted by as much as ±50%.

**FILM THICKNESS (CAPILLARY GC):** Can be adjusted by as much as −50% to 100%.

**Particle size (HPLC):** For isocratic separations, the particle size and/or the length of the column may be modified provided that the ratio of the column length (L) to the particle size (dp) remains constant or into the range between −25% and 50% of the prescribed L/dp ratio. Alternatively (as for the application of particle-size adjustment to superficially porous particles), other combinations of L and dp can be used provided that the number of theoretical plates (N) is within −25% to 50%, relative to the prescribed column. Caution should be used when the adjustment results in a higher number of theoretical plates that generate smaller peak volumes, which may require adjustments to minimize extra-column band broadening by factors such as instrument plumbing, detector cell volume and sampling rate, and injection volume. For gradient separations, changes in length, column inner diameter, and particle size are not allowed.

**Particle size (GC):** Changing from a larger to a smaller or from a smaller to a larger particle size GC mesh support is acceptable if the chromatography meets the requirements of system suitability and the same particle size range ratio is maintained. The particle size range ratio is defined as the diameter of the largest particle divided by the diameter of the smallest particle.

**Flow rate (GC):** The flow rate can be adjusted by as much as ±50%. [Note—When the monograph specifies a linear velocity parameter, the allowed velocity adjustment is between +50% and −25%, provided the carrier gas system can be maintained under control at the desired set points.]

**Flow rate (HPLC):** When the particle size is changed, the flow rate may require adjustment, because smaller-particle columns will require higher linear velocities for the same performance (as measured by reduced plate height). Flow rate changes for both a change in column diameter and particle size can be made by:

\[
F_2 = F_1 \times \left[\frac{(dc_1^2 \times dp_1)}{(dc_2^2 \times dp_2)}\right]
\]

where \(F_1\) and \(F_2\) are the flow rates for the original and modified conditions, respectively, \(dc_1\) and \(dc_2\) are the respective column diameters, and \(dp_1\) and \(dp_2\) are the particle sizes.

When a change is made from ≥3-µm to <3-µm particles in isocratic separations, an additional increase in linear velocity (by adjusting flow rate) may be justified, provided that the column efficiency does not drop by >20%. Similarly, a change from <3-µm to ≥3-µm particles may require additional reduction of linear velocity (flow rate) to avoid reduction in column efficiency by >20%. Changes in \(F, dc,\) and \(dp\) are not allowed for gradient separations.

Additionally, the flow rate can be adjusted by ±50% (isocratic only).

**EXAMPLES:** Adjustments in column length, internal diameter, particle size, and flow rate can be used in combination to give equivalent conditions (same N), but with differences in pressure and run time. Table 2 lists some of the more popular column configurations to give equivalent efficiency (N), by adjusting these variables.

<table>
<thead>
<tr>
<th>Table 2. Column Configurations</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Length (L, mm)</strong></td>
</tr>
<tr>
<td><strong>L/dp</strong></td>
</tr>
<tr>
<td>250</td>
</tr>
<tr>
<td>150</td>
</tr>
</tbody>
</table>
### Table 2. Column Configurations (continued)

<table>
<thead>
<tr>
<th>Length (L, mm)</th>
<th>Column Diameter (dc, mm)</th>
<th>Particle Size (dp, µm)</th>
<th>Relative Values</th>
<th>Pressure</th>
<th>Run Time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>L/dp</td>
<td>F</td>
<td>N</td>
</tr>
<tr>
<td>150</td>
<td>2.1</td>
<td>5</td>
<td>30,000</td>
<td>0.2</td>
<td>1.0</td>
</tr>
<tr>
<td>100</td>
<td>4.6</td>
<td>3.5</td>
<td>28,600</td>
<td>1.4</td>
<td>1.0</td>
</tr>
<tr>
<td>100</td>
<td>2.1</td>
<td>3.5</td>
<td>28,600</td>
<td>0.3</td>
<td>1.0</td>
</tr>
<tr>
<td>75</td>
<td>4.6</td>
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<td>30,000</td>
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<td>75</td>
<td>2.1</td>
<td>2.5</td>
<td>30,000</td>
<td>0.4</td>
<td>1.0</td>
</tr>
<tr>
<td>50</td>
<td>4.6</td>
<td>1.7</td>
<td>29,400</td>
<td>2.9</td>
<td>1.0</td>
</tr>
<tr>
<td>50</td>
<td>2.1</td>
<td>1.7</td>
<td>29,400</td>
<td>0.6</td>
<td>1.0</td>
</tr>
</tbody>
</table>

For example, if a monograph specifies a 150-mm × 4.6-mm; 5-µm column operated at 1.5 mL/min, the same separation may be expected with a 75-mm × 2.1-mm; 2.5-µm column operated at 1.5 mL/min × 0.4 = 0.6 mL/min, along with a pressure increase of about four times and a reduction in run time to about 30% of the original.

**Injection volume (HPLC):** The injection volume can be adjusted as far as it is consistent with accepted precision, linearity, and detection limits. Note that excessive injection volume can lead to unacceptable band broadening, causing a reduction in N and resolution, which applies to both gradient and isocratic separations.

**Injection volume and split volume (GC):** The injection volume and split volume may be adjusted if detection and repeatability are satisfactory.

**Column temperature (HPLC):** The column temperature can be adjusted by as much as ±10°. Column thermostating is recommended to improve control and reproducibility of retention time, which applies to both gradient and isocratic separations.

**Oven temperature (GC):** The oven temperature can be adjusted by as much as ±10%.

**Oven temperature program (GC):** Adjustment of temperatures is permitted as stated above. When the specified temperature must be maintained or when the temperature must be changed from one value to another, an adjustment of up to ±20% is permitted.

Unless otherwise directed in the monograph, system suitability parameters are determined from the analyte peak.

Measured values of R_r, R_F, or t_R for the sample substance do not deviate from the values obtained for the reference compound and mixture by more than the statistically determined reliability estimates from replicate assays of the reference compound. RRT may be provided in monographs for informational purposes only to aid in peak identification. There are no acceptance criteria applied to RRT.

Suitability testing is used to ascertain the effectiveness of the final operating system, which should be subjected to this testing. Make injections of the appropriate preparation(s), as required, in order to demonstrate adequate system suitability (as described in the Chromatographic system section of the method in a monograph) throughout the run.

The preparation can be a standard preparation or a solution containing a known amount of analyte and any additional materials (e.g., excipients or impurities) useful in controlling the analytical system. Whenever there is a significant change in the chromatographic system (equipment, mobile phase component, or other components) or in a critical reagent, system suitability is to be reestablished. No sample analysis is acceptable unless the suitability of the system has been demonstrated.

### QUANTITATION

During quantitation, disregard peaks caused by solvents and reagents or arising from the mobile phase or the sample matrix.

In the linear range, peak areas and peak heights are usually proportional to the quantity of compound eluting. The peak areas and peak heights are commonly measured by electronic integrators but may be determined by more classical approaches. Peak areas are generally used but may be less accurate if peak interference occurs. The components measured are separated from any interfering components. Peak tailing and fronting is minimized, and the measurement of peaks on tails of other peaks are avoided when possible.

Although comparison of impurity peaks with those in the chromatogram of a standard at a similar concentration is preferred, impurity tests may be based on the measurement of the peak response due to impurities and expressed as a percentage of the area of the drug peak. The standard may be the drug itself at a level corresponding to, for example, 0.5% impurity, assuming similar peak responses. When impurities must be determined with greater certainty, use a standard of the impurity itself or apply a correction factor based on the response of the impurity relative to that of the main component.

**External Standard Method**

The concentration of the component(s) quantified is determined by comparing the response(s) obtained with the sample solution to the response(s) obtained with a standard solution.

**Internal Standard Method**

Equal amounts of the internal standard are introduced into the sample solution and a standard solution. The internal standard is chosen so that it does not react with the test material and does not contain impurities with the same retention time as that...
of the analytes, and is stable and resolved from the component(s) quantified (analytes). The concentrations of the analytes are determined by comparing the ratios of their peak areas or peak heights and the internal standard in the sample solution with the ratios of their peak areas or peak heights and the internal standard in the standard solution.

**Normalization Procedure**

The percent content of a component of the test material is calculated by determining the area of the corresponding peak as a percentage of the total area of all the peaks, excluding those due to solvents or reagents or arising from the mobile phase or the sample matrix and those at or below the limit at which they can be disregarded.

**Calibration Procedure**

The relationship between the measured or evaluated signal $y$ and the quantity (e.g., concentration or mass) of substance $x$ is determined, and the calibration function is calculated. The analytical results are calculated from the measured signal or evaluated signal of the analyte and its position on the calibration curve.

In tests for impurities for both the external standard method, when a dilution of the sample solution is used for comparison, and the normalization procedure, any correction factors indicated in the monograph are applied (e.g., when the relative response factor is outside the range of 0.8–1.2).

When the impurity test prescribes the total of impurities or there is a quantitative determination of an impurity, choice of an appropriate threshold setting and appropriate conditions for the integration of the peak areas is important. In such tests the limit at or below which a peak is disregarded is generally 0.05%. Thus, the threshold setting of the data collection system corresponds to at least half of this limit. Integrate the peak area of any impurity that is not completely separated from the principal peak, preferably by valley-to-valley extrapolation (tangential skim).

**Add the following:**

**630** VISUAL COMPARISON

The purpose of this test is to provide the details for the visual comparison of the color and/or turbidity of sample solutions of certain concentration to a standard solution or a series of standard solutions of known concentration. Where a color or turbidity comparison is directed, follow the procedures and conditions outlined below for performing these tests.

**Comparison vessels:** Color-comparison tubes matched as closely as possible in internal diameter, in depth of sample solution, and in all other respects should be used.

**Viewing conditions for turbidity comparison:** Tubes should be viewed horizontally against a dark background with the aid of a light source directed from the sides of the tubes.

**Viewing conditions for color comparison:** Tubes should be viewed downward against a white background. Most of the time, common room lighting is sufficient to perform the assessment. A light source directed from beneath the bottoms of the tubes may be used if needed and if the practice is consistent between the materials under comparison.▲ (USP 1-May-2019)

**643** TOTAL ORGANIC CARBON

Total organic carbon (TOC) is an indirect measure of organic molecules present in pharmaceutical waters measured as carbon. Organic molecules are introduced into the water from the source water, from purification and distribution system materials, from biofilm growing in the system, and from the packaging of sterile and nonsterile waters. TOC can also be used as a process control attribute to monitor the performance of unit operations comprising the purification and distribution system. A TOC measurement is not a replacement test for endotoxin or microbiological control. Although there can be a qualitative relationship between a food source (TOC) and microbiological activity, there is no direct numerical correlation.

A number of acceptable methods exist for analyzing TOC. This chapter does not endorse, limit, or prevent any technologies from being used, but this chapter provides guidance on how to qualify these analytical technologies for use as well as guidance on how to interpret instrument results for use as a limit test.

Apparatuses commonly used to determine TOC in water for pharmaceutical use have in common the objective of oxidizing the organic molecules in the water to produce carbon dioxide followed by the measurement of the amount of carbon dioxide produced. Then the amount of CO$_2$ produced is determined and used to calculate the organic carbon concentration in the water.

All technologies must discriminate between the inorganic carbon, which may be present in the water from sources such as dissolved CO$_2$ and bicarbonate, and the CO$_2$ generated from the oxidation of organic molecules in the sample. The discrimination may be accomplished either by determining the inorganic carbon and subtracting it from the total carbon (total carbon is the sum of organic carbon and inorganic carbon), or by purging inorganic carbon from the sample before oxidation. Although purging may entrain organic molecules, such purgeable organic carbon is present in negligible quantities in water for pharmaceutical use.

**PROCEDURES**

• **BULK WATER**

The following sections apply to tests for bulk Purified Water, Water for Injection, Water for Hemodialysis, and the condensate of Pure Steam.
Apparatus requirements: This test method is performed either as an on-line test or as an off-line laboratory test using a calibrated instrument. The suitability of the apparatus must be periodically demonstrated as described below. In addition, it must have a manufacturer’s specified limit of detection of 0.05 mg/L (0.05 ppm) or lower of carbon. When testing water for quality control purposes, ensure that the instrument and its data are under appropriate control and that the sampling approaches and locations of both on-line and off-line measurements are representative of the quality of the water used. The nature of the water production, distribution, and use should be considered when selecting either on-line or off-line measurement.

Reagent water: Use water having a TOC level of not more than 0.10 mg/L. [NOTE—A conductivity requirement may be necessary in order to ensure method reliability.]

Container preparation: Organic contamination of containers results in higher TOC values. Therefore, use labware and containers that have been scrupulously cleaned of organic residues. Any method that is effective in removing organic matter can be used (see Cleaning Class Apparatus (1051)). Use Reagent water for the final rinse.

Standard solution: Unless otherwise directed in the individual monograph, dissolve in the Reagent water an accurately weighed quantity of USP Sucrose RS to obtain a solution having a concentration of 1.19 mg/L of sucrose (0.50 mg/L of carbon).

System suitability solution: Dissolve in Reagent water an accurately weighed quantity of USP 1,4-Benzquinone RS to obtain a solution having a concentration of 0.75 mg/L (0.50 mg/L of carbon).

Reagent water control: Use a suitable quantity of Reagent water obtained at the same time as that used in the preparation of the Standard Solution and the System suitability solution.

Water sample: Obtain an on-line or off-line sample that suitably reflects the quality of water used.

Other control solutions: Prepare appropriate reagent blank solutions or other specified solutions needed for establishing the apparatus baseline or for calibration adjustments following the manufacturer’s instructions, and run the appropriate blanks to zero the instrument, if necessary.

System suitability: Test the Reagent water control in the apparatus, and record the response, \( r_w \). Repeat the test using the Standard solution, and record the response, \( r_s \). Calculate the corrected Standard solution response, which is also the limit response, by subtracting the Reagent water control response from the response of the Standard solution. The theoretical limit of 0.50 mg/L of carbon is equal to the corrected Standard solution response, \( r_s - r_w \). Test the System suitability solution in the apparatus, and record the response, \( r_{SS} \). Calculate the corrected System suitability solution response by subtracting the Reagent water control response from the response of the System suitability solution, \( r_{SS} - r_w \). Calculate the percent response efficiency for the System suitability solution:

\[
\text{% response efficiency} = 100 \left( \frac{r_{SS} - r_w}{r_s - r_w} \right)
\]

\( r_{SS} \) = instrument response to the System suitability solution

\( r_w \) = instrument response to the Reagent water control

\( r_s \) = instrument response to the Standard solution

The system is suitable if the percent response efficiency is not less than 85% and not more than 115%.

Procedure: Perform the test on the Water Sample, and record the response, \( r_w \). The Water Sample meets the requirements if \( r_s \) is not more than the limit response, \( r_s - r_w \). This method can be performed using on-line or off-line instrumentation that meets the Apparatus requirements.

**STERILE WATER**

The following sections apply to tests for Sterile Water for Injection, Sterile Purified Water, Sterile Water for Irrigation, and Sterile Water for Inhalation.

Follow the requirements in Bulk Water, with the following exceptions.

Apparatus requirements: In addition to the Apparatus requirements in Bulk Water, the apparatus must have a manufacturer’s specified limit of detection of 0.10 mg/L (0.10 ppm) or lower of carbon.

Reagent water: Use water having a TOC level of not more than 0.50 mg/L. [NOTE—A conductivity requirement may be necessary in order to ensure method reliability.]

Standard solution: Unless otherwise directed in the individual monograph, dissolve in the Reagent water an accurately weighed quantity of USP Sucrose RS to obtain a solution having a concentration of 19.0 mg/L of sucrose (8.0 mg/L of carbon).

System suitability solution: Dissolve in Reagent water an accurately weighed quantity of USP 1,4-Benzquinone RS to obtain a solution having a concentration of 12.0 mg/L (8.0 mg/L of carbon).

Water sample: Obtain a sample that suitably reflects the quality of water used. Before opening, vigorously agitate the package to homogenize the water sample. Several packages may be required in order to collect sufficient water for analysis.

System suitability: Test the Reagent water control in the apparatus, and record the response, \( r_w \). Repeat the test using the Standard solution, and record the response, \( r_s \). Calculate the corrected Standard solution response, which is also the limit response, by subtracting the Reagent water control response from the response of the Standard solution. The theoretical limit of 8.0 mg/L of carbon is equal to the corrected Standard solution response, \( r_s - r_w \). Test the System suitability solution in the apparatus, and record the response, \( r_{SS} \). Calculate the corrected System suitability solution response by subtracting the Reagent water control response from the response of the System suitability solution, \( r_{SS} - r_w \). Calculate the percent response efficiency for the System suitability solution:

\[
\text{% response efficiency} = 100 \left( \frac{r_{SS} - r_w}{r_s - r_w} \right)
\]

\( r_{SS} \) = instrument response to the System suitability solution

\( r_w \) = instrument response to the Reagent water control

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\( r_s = \text{instrument response to the Standard solution} \)

The system is suitable if the percent response efficiency is not less than 85% and not more than 115%.

**Procedure:** Perform the test on the Water Sample, and record the response, \( r_p \). The Water sample meets the requirements if \( r_p \) is not more than the limit response, \( r_s - r_m \), determined in the System suitability requirements in Sterile Water.

### ADDITIONAL REQUIREMENTS

- **USP Reference Standards (11)**
  - USP 1,4-Benzoquinone RS
  - USP Sucrose RS

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### 645 WATER CONDUCTIVITY

#### INTRODUCTION

Electrical conductivity in water is a measure of the ion-facilitated electron flow through it. Water molecules dissociate into ions as a function of pH and temperature and result in a very predictable conductivity. Some gases, most notably carbon dioxide, readily dissolve in water and interact to form ions, which predictably affect conductivity also. For the purpose of this discussion, these ions and their resulting conductivity can be considered intrinsic to the water.

Water conductivity is also affected by the presence of extraneous ions. The extraneous ions used in modeling the conductivity specifications described below are the chloride and ammonia ions. The conductivity of the ubiquitous chloride ion (at the theoretical endpoint concentration of 0.47 ppm when chloride was a required attribute test in USP 22 and earlier revisions) and the ammonium ion (at the limit of 0.3 ppm) represents a major portion of the allowed water ionic impurity level. A balancing quantity of anions (such as chloride, to counter the ammonium ion) and cations (such as sodium, to counter the chloride ion) is included in this allowed impurity level to maintain electroneutrality. Extraneous ions such as these may have a significant effect on the water's chemical purity and suitability for use in pharmaceutical applications.

The procedure in the section Bulk Water is specified for measuring the conductivity of waters such as Purified Water, Water for Injection, Water for Hemodialysis, and the condensate of Pure Steam. The procedure in the section Sterile Water is specified for measuring the conductivity of waters such as Sterile Purified Water, Sterile Water for Injection, Sterile Water for Inhalation, and Sterile Water for Irrigation.

The procedures below shall be performed using instrumentation that has been calibrated, has conductivity sensor cell constants that have been accurately determined, and has a temperature compensation function that has been disabled for Bulk Water Stage 1 testing. For both online and offline measurements, the suitability of instrumentation for quality control testing is also dependent on the sampling location(s) in the water system. The selected sampling instrument location(s) must reflect the quality of the water used.

### INSTRUMENT SPECIFICATIONS AND OPERATING PARAMETERS

Water conductivity must be measured accurately with calibrated instrumentation. An electrical conductivity measurement consists of the determination of the conductance, \( G \) (or its inverse, resistance, \( R \)), of the fluid between and around the electrodes. The conductance (1/\( R \)) is directly affected by the geometrical properties of the electrodes; i.e., the conductance is inversely proportional to the distance (\( d \)) between the electrodes and proportional to the area (\( A \)) of the electrodes. This geometrical ratio (\( d/A \)) is known as the cell constant, \( \Theta \). Thus the measured conductance is normalized for the cell constant to determine the conductivity, \( \kappa \), according to the following equation:

\[
\kappa \text{ (S/cm)} = \Theta \text{ (cm}^{-1}\text{)}/R \text{ (}\Omega\text{)}
\]

It is the cell constant and the resistance measurement that must be verified and adjusted, if necessary.

#### Cell Constant

The cell constant must be known within ±2%. The cell constant can be verified directly by using a solution of known or traceable conductivity, or indirectly by comparing the instrument reading taken with the conductivity sensor in question to readings from a conductivity sensor of known or traceable cell constant. If necessary, adjust the cell constant following the manufacturer’s instrument protocol. The frequency of verification/calibration is a function of the sensor design.

#### Resistance Measurement

Calibration (or verification) of the resistance measurement is accomplished by replacing the conductivity sensor electrodes with precision resistors having standards traceable to NIST or equivalent national authorities in other countries (accurate to ±0.1% of the stated value) to give a predicted instrument conductivity response. The accuracy of the resistance measurement is acceptable if the measured conductivity with the traceable resistor is within ±0.1 µS/cm of the calculated value according to the equation above. For example, the traceable resistor is 50 kΩ, and the cell constant, \( \Theta \), is 0.10 cm⁻¹. The calculated value is 2.0 × 10⁻⁶ S/cm or 2.0 µS/cm. The measured value should be 2.0 ± 0.1 µS/cm. The instrument must have a minimum resolution of 0.1 µS/cm on the lowest range.
The target conductivity value(s) should be based on the type of water to be analyzed, and it should be equal to or less than the water conductivity limit for that type of water. Multiple measuring circuits may be embedded in the meter or the sensor, and each circuit may require separate verification or calibration before use. The frequency of recalibration is a function of instrument system design.

**System Verification**

The cell constant of the user’s sensor can be determined with the user’s resistance measurement system, or the cell constant can be determined with an independent resistance measurement system. If the cell constant is determined with an independent resistance measurement system, it is recommended that the user verify that the sensor has been properly connected to the resistance measurement system to ensure proper performance. Verification can be made by comparing the conductivity (or resistivity) values displayed by the measuring equipment with those of an external calibrated conductivity-measuring device. The two non–temperature-compensated conductivity (or resistivity) values should be equivalent to or within ±5% of each other, or should have a difference that is acceptable on the basis of product water criticality and/or the water conductivity ranges in which the measurements are taken. The two conductivity sensors should be positioned close enough together to measure the same water sample at the same temperature and water quality.

**Temperature Compensation and Temperature Measurements**

Because temperature has a substantial effect on conductivity readings of specimens at high and low temperatures, many instruments automatically correct the actual reading to display the value that theoretically would be observed at the nominal temperature of 25°. This is typically done using a temperature sensor embedded in the conductivity sensor and a software algorithm embedded in the instrument. This temperature compensation algorithm may not be accurate for the various water types and impurities. For this reason, conductivity values used in the *Stage 1* test for *Bulk Water* are non–temperature-compensated measurements. Other conductivity tests that are specified for measurement at 25° can use either temperature-compensated or non–temperature-compensated measurements.

A temperature measurement is required for the *Stage 1* test or for the other tests at 25°. It may be made using the temperature sensor embedded in the conductivity cell sensor. An external temperature sensor positioned near the conductivity sensor is also acceptable. Accuracy of the temperature measurement must be ±2°.

**BULK WATER**

The procedure and test limits in this section are intended for *Purified Water*, *Water for Injection*, *Water for Hemodialysis*, the condensate of *Pure Steam*, and any other monographs that specify this section. This is a three-stage test method to accommodate online or offline testing. Online conductivity testing provides real-time measurements and opportunities for real-time process control, decision, and intervention. Precautions should be taken while collecting water samples for offline conductivity measurements. The sample may be affected by the sampling method, the sampling container, and environmental factors such as ambient carbon dioxide concentration and organic vapors. This procedure can be started at *Stage 2* if offline testing is preferred.

**Procedure**

**STAGE 1**

*Stage 1* is intended for online measurement or may be performed offline in a suitable container.

1. Determine the temperature of the water and the conductivity of the water with a non–temperature-compensated conductivity reading.
2. Using Table 1, find the temperature value that is NMT the measured temperature, i.e., the next lower temperature. The corresponding conductivity value on this table is the limit.
   
   **[NOTE—Do not interpolate.]**
3. If the measured conductivity is NMT the table value determined in step 2, the water meets the requirements of the test for conductivity. If the conductivity is higher than the table value, proceed with *Stage 2*.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Conductivity Requirement (µS/cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.6</td>
</tr>
<tr>
<td>5</td>
<td>0.8</td>
</tr>
<tr>
<td>10</td>
<td>0.9</td>
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<tr>
<td>15</td>
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<td>20</td>
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<tr>
<td>25</td>
<td>1.3</td>
</tr>
<tr>
<td>30</td>
<td>1.4</td>
</tr>
</tbody>
</table>

Published on March 26, 2020
Table 1. Stage 1—Temperature and Conductivity Requirements (for non–temperature-compensated conductivity measurements only) (continued)

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Conductivity Requirement (µS/cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>35</td>
<td>1.5</td>
</tr>
<tr>
<td>40</td>
<td>1.7</td>
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<tr>
<td>45</td>
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<td>95</td>
<td>2.9</td>
</tr>
<tr>
<td>100</td>
<td>3.1</td>
</tr>
</tbody>
</table>

STAGE 2

4. Transfer a sufficient amount of water to a suitable container, and stir the test specimen. Adjust the temperature, if necessary, and, while maintaining it at 25 ± 1°C, begin vigorously agitating the test specimen while periodically observing the conductivity. When the change in conductivity (due to uptake of atmospheric carbon dioxide) is less than a net of 0.1 µS/cm per 5 min, note the conductivity.

[NOTE—Conductivity measurements at this stage may be temperature-compensated to 25°C or non–temperature-compensated.]

5. If the conductivity is not greater than 2.1 µS/cm, the water meets the requirements of the test for conductivity. If the conductivity is greater than 2.1 µS/cm, proceed with Stage 3.

STAGE 3

6. Perform this test within approximately 5 min of the conductivity determination in step 5, while maintaining the sample temperature at 25 ± 1°C. Add a saturated potassium chloride solution to the same water sample (0.3 mL per 100 mL of the test specimen), and determine the pH to the nearest 0.1 pH unit, as directed in pH (791).

7. Referring to Table 2, determine the conductivity limit at the measured pH value. If the measured conductivity in step 4 is NMT the table value determined in step 6, the water meets the requirements of the test for conductivity. If either the measured conductivity is greater than this value or the pH is outside the range of 5.0–7.0, the water does not meet the requirements of the test for conductivity.

Table 2. Stage 3—pH and Conductivity Requirements (for atmosphere- and temperature-equilibrated samples only)

<table>
<thead>
<tr>
<th>pH</th>
<th>Conductivity Requirement (µS/cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.0</td>
<td>4.7</td>
</tr>
<tr>
<td>5.1</td>
<td>4.1</td>
</tr>
<tr>
<td>5.2</td>
<td>3.6</td>
</tr>
<tr>
<td>5.3</td>
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<td>2.5</td>
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<tr>
<td>6.3</td>
<td>2.4</td>
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</table>

Published on March 26, 2020
Table 2. Stage 3—pH and Conductivity Requirements (for atmosphere- and temperature-equilibrated samples only) (continued)

<table>
<thead>
<tr>
<th>pH</th>
<th>Conductivity Requirement (µS/cm)</th>
</tr>
</thead>
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<tr>
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<td>6.9</td>
<td>3.8</td>
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<tr>
<td>7.0</td>
<td>4.6</td>
</tr>
</tbody>
</table>

**STERILE WATER**

The procedure and test limits are intended for Sterile Purified Water, Sterile Water for Injection, Sterile Water for Inhalation, and Sterile Water for Irrigation, and any other monographs that specify this section. The sterile waters are derived from Purified Water or Water for Injection, and therefore have been determined to be compliant with the Bulk Water requirements before being stored in the container. The specification provided represents the maximum allowable conductivity value, taking into consideration the limitation of the measurement method and reasonable container leaching. Such specification and the sampling volume choices should be defined and validated on the basis of the intended purpose of the water.

**Procedure**

Obtain a sample that suitably reflects the quality of water used. Before opening, vigorously agitate the package to homogenize the water sample. Several packages may be required to collect sufficient water for analysis.

Transfer a sufficient amount of water to a suitable container, and stir the test specimen. Adjust the temperature, if necessary, and, while maintaining it at 25 ± 1°, begin vigorously agitating the test specimen while periodically observing the conductivity. When the change in conductivity (due to uptake of ambient carbon dioxide) is less than a net of 0.1 µS/cm per 5 min, note the conductivity.

For containers with a nominal volume of 10 mL or less, if the conductivity is NMT 25 µS/cm, the water meets the requirements. For containers with a nominal volume greater than 10 mL, if the conductivity is NMT 5 µS/cm, the water meets the requirements.

### PACKAGING AND STORAGE REQUIREMENTS

*(A portion of the Packaging section of this chapter will become official on December 1, 2025 (USP 1-Aug-2020) as indicated. Early adoption of the requirements in this chapter and Plastic Materials of Construction (661.1) and Plastic Packaging Systems for Pharmaceutical Use (661.2) is permitted by USP.)*

**INTRODUCTION**

The purpose of this chapter is to provide packaging definitions, auxiliary packaging information, and storage condition definitions relevant to the storage and distribution of active ingredients, excipients, and medical products, such as pharmaceuticals, devices, combination products (e.g., drug-eluting stents), and dietary supplements.

*(Change to read:)*

**PACKAGING**

Packaging materials must not interact physically or chemically with a packaged article in a manner that causes its safety, identity, strength, quality, or purity to fail to conform to established requirements. Any plastic material used to construct a Packaging system must meet the applicable requirements of Plastic Materials of Construction (661.1) and Plastic Packaging Systems for Pharmaceutical Use (661.2). All Packaging systems must meet the applicable requirements specified in Containers—Glass (660), Plastic Packaging Systems and Their Materials of Construction (661), Plastic Packaging Systems for Pharmaceutical Use (661.2), and Auxiliary Packaging Components (670). All elastomeric closures must meet the applicable requirements in Elastomeric Closures for Injections (381). Every monograph in USP–NF must have packaging and storage requirements. For the packaging portion of the statement, the choice of containers is provided in this chapter. For active pharmaceutical ingredients (APIs), the choice would be a tight, well-closed, or, where needed, light-resistant container. For excipients, given their typical presentation as large-volume commodity items (Packaging systems ranging from drums to tank cars), a well-closed container is an appropriate default.
requirement. Articles must be protected from moisture, freezing, and excessive heat (see General Definitions) when no specific directions or limitations are provided.

The compendial requirements for the use of specified containers apply also to articles packaged by Dispensers, Repackers, or other individuals, unless otherwise indicated in the individual drug product monograph.

POISON PREVENTION PACKAGING ACT

This act, which is administered by the United States Consumer Product Safety Commission (CPSC), requires special packaging for most human oral prescription drugs, oral controlled drugs, certain non-oral prescription drugs, certain dietary supplements, and many over-the-counter (OTC) drug preparations, to protect the public from personal injury or illness from misuse of these preparations [16 Code of Federal Regulations (CFR) §1700.14]. The primary packaging of substances regulated under the Poison Prevention Packaging Act (PPPA) must comply with the special packaging standards (16 CFR §1700.15). These apply to all packaging types, including reclosable, non-reclosable, and unit-dose types.

Special packaging is not required for drugs dispensed within a hospital setting for inpatient administration. Also, special packaging does not need to be used by manufacturers and packagers of bulk-packaged prescription drugs that will be repackaged by the pharmacist. PPPA-regulated prescription drugs may be dispensed in non-Child-resistant packaging upon the request of the purchaser or when directed in a legitimate prescription [15 United States Code (USC) §1473].

Manufacturers or packagers of PPPA-regulated OTC preparations are allowed to package one size in non-Child-resistant packaging as long as popular-size, special packages are also supplied. The non-Child-resistant packaging requires special labeling (16 CFR §1700.5).

TEMPERATURE AND STORAGE

Specific directions are stated in some monographs with respect to storage conditions (e.g., the temperature or humidity) at which an article must be stored and shipped. Such directions apply except where the label on the article has different storage conditions that are based on stability studies. Where no specific directions or limitations are provided in the article’s labeling, articles must be protected from moisture, freezing, and excessive heat, and, where necessary, from light during shipping and distribution. Drug substances are exempt from this standard.

Change to read:

GENERAL DEFINITIONS

Packaging Definitions

Packaging system (also referred to as a Container–closure system): The sum of packaging components and materials that together contain and protect the article. This includes Primary packaging components as well as Secondary packaging components when such components are required to provide additional protection.

Container: A receptacle that holds an intermediate compound, API, excipient, or dosage form, and is in direct contact with the article (e.g., ampules, vials, bottles, syringes, and pen injectors).

Closure: A material that seals an otherwise open space of a Container and provides protection for the contents. It also provides access to the contents of the Container (e.g., screw caps and stoppers).

Packaging component: Any single part of the Container–closure system, including: the Container (e.g., ampules, syringes, vials, and bottles); Closures (e.g., screw caps and stoppers); ferrules and overseals; Closure liners (e.g., tube cartridge liners); inner seals; administration ports; overwraps; administration accessories; labels; cardboard boxes; and shrink wrap.

Primary packaging component: A Packaging component that is in direct contact with or may come into direct contact with the article.

Secondary packaging component: A Packaging component that is in direct contact with a Primary packaging component and may provide additional protection for the article.

Tertiary packaging component: A Packaging component that is in direct contact with a Secondary packaging component and may provide additional protection for the article during transportation and/or storage.

Ancillary component: A component or entity that may come into contact with a Tertiary packaging component during the distribution, storage, and/or transportation of the packaged article (e.g., pallets, skids, and shrink wrap).

Associated component: A Packaging component that is typically intended to deliver the drug article to the patient but is not stored in contact with the article for its entire shelf life (e.g., spoons, Dosing cups, and dosing syringes).

Materials of construction: The materials (e.g., glass, plastic, elastomers, and metal) of which a Packaging component consists.

Small-volume injection (also referred to as Small-volume parenteral): An injectable dosage form that is packaged in Containers labeled as containing 100 mL or less.

Large-volume injection (also referred to as Large-volume parenteral): An injectable dosage form that is packaged in Containers labeled as containing more than 100 mL.

Child-resistant packaging: A Packaging system designed or constructed to meet CPSC standards pertaining to opening by children (16 CFR §1700.20 et seq. and 16 CFR §1700.15).

Senior-friendly packaging: A Packaging system designed or constructed to meet CPSC standards pertaining to opening by senior adults (16 CFR §1700.15 and 16 CFR §1700.20).
Restricted delivery system: A Packaging system designed or constructed to restrict (control) the amount of the drug product that may be delivered in order to limit unintended access by children and other similarly vulnerable populations. Restricted delivery systems should meet and may exceed CPSC standards for special packaging [Child-resistant and Senior-friendly packaging (16 CFR §1700.15 et seq.).] For oral medicinal liquids, surface and flow characteristics vary. It is the responsibility of the manufacturer to ensure that all components of the Restricted delivery system provide the intended safety protection. One component of the Restricted delivery system is the flow restrictor, which is a Packaging component that restricts the flow of liquid. The flow restrictor may be used as part of a Restricted delivery system or as an adaptor to facilitate use of a measuring device for oral medicinal liquids. A flow restrictor should not compromise CPSC standards for special packaging [Child-resistant and Senior-friendly packaging (16 CFR §1700.15 et seq.).]

Tamper-evident packaging: A Packaging system that may not be accessed without obvious destruction of the seal or some portion of the Packaging system. Tamper-evident packaging must be used for sterile drug products intended for ophthalmic or otic use, except where extemporaneously compounded for immediate dispensing on prescription. Drug products intended for sale without prescription are also required to comply with the Tamper-evident packaging and labeling requirements of the FDA where applicable (21 CFR §221.132). Preferably, the immediate Container and/or the outer Container or protective packaging used by a manufacturer or distributor for all dosage forms that are not specifically exempt is designed to show evidence of any tampering with the contents.

Reclosable packaging: A package that has been initially opened is capable of being reclosed with a similar degree of security and is capable of being used a sufficient number of times to dispense the total contents without loss of security. Reclosable packaging may incorporate child-resistance capabilities.

Non-reclosable packaging: A package or part of a package that cannot be closed again after all or part of the contents have been removed. Examples of Non-reclosable packaging are blisters, sachets, strips, and other Single-unit containers. Non-reclosable packaging may include cold-formed foil blisters, foil strip packs, and polyvinyl chloride (PVC)/Aclar combining multilayer materials that are thermo-formed or cold-formed foil blisters. Non-reclosable packaging may be child resistant depending on the intended use and place of use. Household non-reclosables are subject to the PPPA as defined in 16 CFR §1700.14.

Hermetic container: A Container–closure system that is impervious to air or any other gas under the ordinary or customary conditions of handling, shipment, storage, and distribution.

Light container: A Container–closure system that protects the contents from contamination by extraneous solids, or vapors; from loss of the article; and from efflorescence, deliquescence, or evaporation under the ordinary or customary conditions of handling, shipment, storage, and distribution, and is capable of tight reclosure. Where a tight container is specified, it may be replaced by a hermetic container for a single dose of an article. [NOTE—Where packaging and storage in a tight container or well-closed container is specified in the individual monograph, the container used for an article when dispensed on prescription meets the requirements in Containers—Performance Testing (671).]

Well-closed container: A Container–closure system that protects the contents from contamination by extraneous solids and from loss of the article under the ordinary or customary conditions of handling, shipment, storage, and distribution. See (671).

Light-resistant container: A Container–closure system that protects the contents from the effects of light by virtue of the specific properties of the material of which it is composed, including any coating applied to it. A clear and colorless or translucent container may be made light-resistant by means of an opaque covering or by use of secondary packaging, in which case the label of the container bears a statement that the opaque covering or secondary packaging is needed until the articles are to be used or administered. Where it is directed to “protect from light” in an individual monograph, preservation in a light-resistant container is intended. See Plastic Packaging Systems for Pharmaceutical Use (661.2), Functionality, Spectral Transmission Requirements for Light-Resistant Components and Systems (USP 1-Aug-2020)

Equivalent container–closure system: A Container–closure system that is as protective as or more protective than the original manufacturer’s Packaging system in terms of moisture vapor transmission rate, oxygen transmission, light transmission, and compatibility. System equivalency extends to any special protective materials, such as those for seals or desiccants associated with the original Packaging system.

Table 1. Packaging Systems Definitions: Injection versus Noninjection

<table>
<thead>
<tr>
<th>Injection</th>
<th>Noninjection</th>
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<tbody>
<tr>
<td>Multiple-dose</td>
<td>Multiple-unit</td>
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<tr>
<td>Single-dose</td>
<td>Single-unit</td>
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<tr>
<td>—</td>
<td>Unit-dose</td>
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<tr>
<td>—</td>
<td>Unit-of-use</td>
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<tr>
<td>Pharmacy bulk package</td>
<td></td>
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<tr>
<td>Imaging bulk package</td>
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</table>

Injection Packaging Systems

Multiple-dose container (also referred to as Multi-dose): A Container–closure system that holds a sterile medication for parenteral administration (injection or infusion) that has met antimicrobial effectiveness testing requirements, or is excluded from such testing requirements by FDA regulation. A Multiple-dose container is intended to contain more than one dose of a drug product. When space permits, a Multiple-dose container is labeled as such. Multiple-dose containers are generally expected to contain 30 mL or less of medications. The beyond-use date for an opened or entered (e.g., needle-punctured) Multiple-dose container is 28 days unless otherwise specified by the manufacturer on the label. An example of a Multiple-dose container is a vial.
- **Single-dose container**: A Container–closure system that holds a sterile medication for parenteral administration (injection or infusion) that is not required to meet the antimicrobial effectiveness testing requirements. A single-dose container is designed for use with a single patient as a single injection/infusion. When space permits, a single-dose container is labeled as such and should include on the label appropriate discard statements. Examples of single-dose containers are vials, ampules, and prefilled syringes.

- **Pharmacy bulk package**: A Container–closure system of a sterile preparation for parenteral use that contains many single doses. The contents are intended for use in a pharmacy admixture program and are restricted to the preparation of admixtures for infusion or, through a sterile transfer device, for the filling of empty sterile syringes. The closure must be penetrated only one time after constitution, if necessary, with a suitable sterile transfer device or dispensing set that allows measured dispensing of the contents. The pharmacy bulk package is to be used only in a suitable work area such as a laminar flow hood (or an equivalent clean-air compounding area). Designation as a pharmacy bulk package is limited to injection, for injection, or injectable emulsion dosage forms as defined in Nomenclature (1121), General Nomenclature Forms. The pharmacy bulk packages, although containing more than one single dose, are exempt from the multiple-dose container volume limit of 30 mL and the requirement that they contain a substance or suitable mixture of substances to prevent the growth of microorganisms. See Labeling (7) for labeling requirements.

- **Imaging bulk package**: A container of a sterile preparation for parenteral use that contains many single doses of a contrast agent (medical imaging drug product) for use with a medical imaging device. The contents are restricted to use in direct conjunction with a device with features to mitigate the risk of cross-contamination (i.e., an automated contrast injection system or contrast management system approved or cleared for use with an imaging bulk package). The sterility assurance of the imaging bulk package contents in part is dependent upon the automated contrast injection system or the contrast management system. The imaging bulk package is to be used only in a room designated for radiological procedures that involve intravascular administration of a contrast agent. Using aseptic technique, the imaging bulk package closure must be penetrated only one time with a suitable sterile component of the automated contrast injection system or contrast management system. If the integrity of the imaging bulk package and the delivery system cannot be assured through direct continuous supervision, the imaging bulk package and all associated disposables for the automated contrast injection system or contrast management system should be discarded.

- **Noninjection Packaging Systems**

  - **Multiple-unit container**: A Container–closure system that permits withdrawal of successive portions of a noninjection article without changing the safety, strength, quality, or purity of the remaining portion (e.g., bottle of capsules, tablets, and oral or topical liquids).

  - **Single-unit container**: A Container–closure system that holds a quantity of a noninjection article intended for administration as a single dose or a single finished device intended for use promptly after the packaging system is opened.

  - **Unit-dose container**: A single-unit Container–closure system for an article intended for administration by other than the parenteral route as a single dose.

  - **Unit-of-use container**: A Container–closure system that contains a specific quantity of an article that is intended to be dispensed as such without further modification except for the addition of appropriate labeling (see (7)). It is not permitted to repackaging Unit-of-use containers for sale.

- **Miscellaneous**

  - **Repackaging**: The act of removing a drug product from the original manufacturer’s packaging system and placing it into another packaging system, usually one of smaller size.

  - **Repackager**: A firm that repackages drug products or medical devices for distribution (e.g., for resale to distributors, hospitals, or pharmacies). For drug products, this applies to a function that is beyond the regular practice of a pharmacy. The distribution is not patient-specific, in that there are no prescriptions. Repackagers and relabelers of medical devices are also required to register and list and meet the provisions described in 21 CFR §807.

  - **Contract packager/contract repackager**: A firm that is contracted by another organization, such as a manufacturer, to package bulk into a marketed container of a drug product. A contract packager does not take ownership from the manufacturer and generally receives the assigned expiration date from the manufacturer.

  - **Dispenser**: A licensed or registered practitioner who is legally responsible for providing the patient with a preparation that is in compliance with a prescription or a medication order and contains a specific patient label. In addition, dispensers may prepare limited quantities in anticipation of a prescription or medication order from a physician. Dispensers are governed by the board of pharmacy of the individual state. The terms “dispenser” and “pharmacy” are used interchangeably.

  - **Beyond-use date**: See (7).

  - **Expiration date**: See (7).

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1 Exceptions may be considered only under conditions described in Pharmaceutical Compounding—Sterile Preparations (797).
Black closure system or black bands: The use of a Black closure system on a vial (e.g., a black cap overseal and a black ferrule to hold the elastomeric closure) or the use of a Black band or series of bands above the constriction on an ampule is prohibited, except for Labeling (7), Labels and Labeling for Injectable Products, Potassium Chloride for Injection Concentrate.

INJECTION PACKAGING

Packaging for sterile products intended for injection must be validated as meeting the containment and protection requirements that are essential for maintaining the article’s quality. Refer to Package Integrity Evaluation—Sterile Products (1207), Package Integrity Testing in the Product Life Cycle—Test Method Selection and Validation (1207.1), Package Integrity Leak Test Technologies (1207.2), and Package Seal Quality Test Technologies (1207.3) for further information regarding sterile product container—closure integrity testing and validation. Closures for Multiple-dose containers permit the withdrawal of the contents without removal or destruction of the Closure. The Closure permits penetration by a needle and, upon withdrawal of the needle, closes at once, protecting the contents against contamination. Refer to (381) for Closure reseal tests that are useful for screening multiple-dose Closures for their reseal properties. Additional testing may be needed to ensure that the specific Closure selected for a product package is able to prevent loss of product contents and microbial contamination under anticipated conditions of multiple entry and use. Piggyback Packaging systems are usually intravenous infusion Container–closure systems that are used to administer a second infusion through a connector of some type or an injection port on the administration set of the first fluid, thereby avoiding the need for another injection site on the patient’s body. Piggyback Packaging systems also are known as secondary infusion containers.

The volume of injection in a Single-dose container provides the amount specified for one-time parenteral administration, and in no case is more than sufficient to permit the withdrawal and administration of 1 L. Preparations intended for intraspinal, intracisternal, or peridural administration are packaged in Single-dose containers only. Unless otherwise specified in the individual monograph, a Multiple-dose container contains a volume of injection sufficient to permit the withdrawal of NMT 30 mL.

The following injections are exempt from the 1-L restriction of the foregoing requirements relating to packaging:

- Injections packaged for extravascular use as irrigation solutions or peritoneal dialysis solutions
- Injections packaged for intravascular use as parenteral nutrition or as replacement or substitution fluid to be administered continuously during hemofiltration

Injections packaged for intravascular use that may be used for intermittent, continuous, or bolus replacement fluid administration during hemodialysis or other procedures, unless excepted above, must conform to the 1-L restriction. Injections labeled for veterinary use are exempt from the packaging and storage requirements concerning the limitation to single-dose Packaging systems and the limitation on the volume of Multiple-dose containers.

Packaging for Constitution

Containers, including the Closures, for dry solids intended for injection must not interact physically or chemically with the preparation in any manner that alters the strength, quality, or purity beyond the official requirements under the ordinary or customary conditions of handling, shipment, storage, sale, and use. A Packaging system for a sterile solid permits the addition of a suitable solvent and withdrawal of portions of the resulting solution or suspension in such manner that the sterility of the product is maintained. Where the assay in a monograph provides a procedure for the sample solution, in which the total withdrawable contents are to be withdrawn from a Single-dose container with a hypodermic needle and syringe, the contents are to be withdrawn as completely as possible into a dry hypodermic syringe of a rated capacity not exceeding 3 times the volume to be withdrawn and fitted with a 21-gauge needle NLT 2.5 cm (1 inch) in length. Care must be taken to expel any air bubbles, and the contents are then discharged into a Container for dilution and assay.

MEDICAL GAS PACKAGING

Gas cylinder: A metallic Packaging system constructed of steel or aluminum and designed to hold medical gases under pressure; these gases may include: Carbon Dioxide USP, Helium USP, Medical Air USP, Nitrous Oxide USP, Nitrogen NF, and Oxygen USP. As a safety measure, for carbon dioxide, helium, medical air, nitrous oxide, and oxygen, the Pin Index Safety System of matched fittings is recommended for cylinders of Size E or smaller.

ASSOCIATED COMPONENTS

Many Associated Components are graduated for measurement and dose administration. Associated Components can be packaged with the drug product or sold and purchased separately. It is the responsibility of the manufacturer to ensure that the appropriate measurement and dosing component is provided or that a general purpose component, such as those described in this section, is specified for delivering the appropriate amount/dose with the intended accuracy. Liquid preparations have unique surface and flow characteristics. Consequently, the volume delivered from a measurement/dosing component may vary for each preparation.

The graduated Associated Components described in this section are for general use and should be composed of safe materials. Graduated markings should be legible, indelible, and on an extraoral surface that does not contact the product.

The associated volume markings must be in metric units only and limited to a single measurement scale that corresponds with the dosing instructions on the OTC or prescription container label (see Prescription Container Labeling (17)). Under expected conditions of use, the volume error incurred in measuring liquids for individual dose administration by means of such graduated components should be NMT 10% of the indicated amount of the liquid preparation with which the graduated component will be used.

Dosing cup: A measuring device consisting of a small cup that may be packaged with oral liquid articles.

Dosing spoon: A measuring device consisting of a bowl and handle that may be packaged with oral liquid articles. The handle may be a graduated tube.
**Temperature and Storage Definitions**

**Freezer:** A place in which the temperature is controlled between −25° and −10° (−13° and 14° F). It is noted that, in some instances, articles may have a recommended storage condition below −20° (−4° F). In such cases, the temperature of the storage location should be controlled to ±10°.

**Refrigerator:** A cold place in which the temperature is controlled between 2° and 8° (36° and 46° F).

**Cold:** Any temperature not exceeding 8° (46° F).

**Cool:** Any temperature between 8° and 15° (46° and 59° F). [Note—An article for which storage in a cool place is directed or recommended may, alternatively, be stored and shipped as refrigerated, unless otherwise specified by the individual monograph.]

**Room temperature** (also referred to as Ambient temperature): The temperature prevailing in a working environment.

**Controlled room temperature:** The temperature maintained thermostatically that encompasses the usual and customary working environment of 20°−25° (68°−77° F). The following conditions also apply.

Mean kinetic temperature not to exceed 25°. Excursions between 15° and 30° (59° and 86° F) that are experienced in pharmacies, hospitals, and warehouses, and during shipping are allowed. Provided the mean kinetic temperature does not exceed 25°, transient spikes up to 40° are permitted as long as they do not exceed 24 h. Spikes above 40° may be permitted only if the manufacturer so instructs.

Articles may be labeled for storage at “controlled room temperature” or at “20°−25°”, or other wording based on the same mean kinetic temperature [see also Good Storage and Distribution Practices for Drug Products (1079), Quality Management System, Environmental Management System, Mean Kinetic Temperature (MKT) Calculation].

An article for which storage at Controlled room temperature is directed may, alternatively, be stored and shipped in a cool place or refrigerated, unless otherwise specified in the individual monograph or on the label.

**Warm:** Any temperature between 30° and 40° (86° and 104° F).

**Excessive heat:** Any temperature above 40° (104° F).

**Dry place:** A place that does not exceed 40% average relative humidity at 20° (68° F) or the equivalent water vapor pressure at other temperatures. The determination may be made by direct measurement at the place. Determination is based on NLT 12 equally spaced measurements that encompass either a season, a year, or, where recorded data demonstrate, the storage period of the article. There may be values of up to 45% relative humidity provided that the average value does not exceed 40% relative humidity. Storage in a Container validated to protect the article from moisture vapor, including storage in bulk, is considered a Dry place.

**Protect from freezing:** The Container label will bear an appropriate instruction to protect the article from freezing in cases where freezing exposes an article to loss of strength or potency or to destructive alteration of its characteristics. These risks are present in addition to the risk that the Container may break if exposed to freezing temperatures.

**Protect from light:** Where light subjects an article to loss of strength or potency or to destructive alteration of its characteristics, the Container label bears an appropriate instruction to protect the article from light. The article must be packaged in a light-resistant Container.

### 〈698〉 Deliverable Volume

**Purpose**

The following tests are designed to provide assurance that oral liquids will, when transferred from the original container, deliver the volume of dosage form that is declared on the label.

**Scope**

These tests are applicable to products that are dispensed by pouring from the container. The tests apply whether the products are supplied as liquid preparations or liquid preparations that are constituted from solids upon the addition of a designated volume of a specific diluent. They are not required for an article packaged in single-unit containers when the monograph includes the test for Uniformity of Dosage Units (905).

**Density Determination**

Because of the tendency of oral liquids to entrain air when shaken or transferred, a more accurate method for determining the delivered volume is to first determine the delivered mass, and then, using the density of the material, to convert the mass to delivered volume. In order to do that, a determination of the density of the material is required. The following is one method to determine density:

1. Tare a 100-mL volumetric flask containing 50.0 mL of water.
2. Add approximately 25 g of well-shaken product, and gently swirl the contents to mix.
3. Reweigh the flask.
4. From a buret, add an accurately measured amount of water to bring the flask contents to volume while gently swirling the contents of the flask. Record the volume taken from the buret.
5. Calculate the density of the sample:

\[ \frac{W}{V} \]

in which \( W \) is the weight, in g, of the material taken; and \( V \) is 50.0 mL minus the volume, in mL, of water necessary to adjust the contents of the flask to volume. Other methods to determine the density may be employed depending on the formulation (e.g., substantially nonaqueous formulations).

**TEST PREPARATIONS**

For the determination of deliverable volume, select NLT 30 containers, and proceed as follows for the dosage form designated.

**Oral Solutions and Oral Suspensions**

Shake the contents of 10 containers individually.

**Powders That Are Labeled to State the Volume of Oral Liquid That Results When the Powder Is Constituted with the Volume of Diluent Stated in the Labeling**

Constitute 10 containers with the volume of diluent stated in the labeling, accurately measured, and shake individually.

**PROCEDURE**

The deliverable volume can be determined by weight as follows:
1. Discharge the container contents into a suitable tared container (allowing drainage for NMT 5 s for single-dose containers and NMT 10 min for multiple-unit containers).
2. Determine the mass of the contents.
3. Calculate the volume using the density.

Alternatively, the following by volume procedure may be used:
1. Under conditions of use or as instructed in the labeling, carefully discharge the contents of each container into separate dry graduated cylinders of a rated capacity not exceeding two and a half times the volume to be measured, and calibrated “to contain” (see Volumetric Apparatus (31)). Care must be taken to avoid the formation of air bubbles during the process. In the absence of labeling instructions, support the containers at about a 30° angle to the horizontal, and gently discharge the contents into the graduated cylinder.
2. Allow each container to drain for a period not to exceed 10 min for multiple-unit containers and 5 s for single-unit containers, unless otherwise specified in the monograph.
3. When free from bubbles, measure the volume of each mixture.

**ACCEPTANCE CRITERIA**

Use the following criteria to determine compliance with this test.

**For Multiple-Unit Containers (see Figure 1)**

The average volume of liquid obtained from the 10 containers is NLT 100%, and the volume of no container is less than 95% of the volume declared in the labeling. If A, the average volume is less than 100% of that declared in the labeling, but the volume of no container is less than 95% of the labeled amount, or if B, the average volume is NLT 100% and the volume of NMT 1 container is less than 95%, but is NLT 90% of the labeled volume, perform the test on 20 additional containers. The average volume of liquid obtained from the 30 containers is NLT 100% of the volume declared in the labeling; and the volume of liquid obtained from NMT 1 of the 30 containers is less than 95%, but NLT 90% of that declared in the labeling.
Figure 1. Decision scheme for multiple-unit containers. (AV = Average volume. LV = Labeled volume)

For Single-Unit Containers (see Figure 2)

The average volume of liquid obtained from the 10 containers is NLT 100%, and the volume of each of the 10 containers lies within the range of 95%–110% of the volume declared in the labeling. If A, the average volume is less than 100% of that declared in the labeling, but the volume of no container is outside the range of 95%–110%, or if B, the average volume is NLT 100% and the volume of NMT 1 container is outside the range of 95%–110%, but within the range of 90%–115%, perform the test on 20 additional containers. The average volume of liquid obtained from the 30 containers is NLT 100% of the volume declared in the labeling; and the volume obtained from NMT 1 of the 30 containers is outside the range of 95%–110%, but within the range of 90%–115% of the volume declared on the labeling.
Figure 2. Decision scheme for single-unit containers. (AV = average volume; LV = labeled volume)

〈711〉 DISSOLUTION

General chapter Dissolution (711) is being harmonized with the corresponding texts of the European Pharmacopoeia and/or the Japanese Pharmacopoeia. These pharmacopeias have undertaken to not make any unilateral change to this harmonized chapter. Portions of the present general chapter text that are national USP text, and therefore not part of the harmonized text, are marked with symbols (◆) to specify this fact.

This test is provided to determine compliance with the dissolution requirements *where stated in the individual monograph, for dosage forms administered orally. In this general chapter, a dosage unit is defined as 1 tablet or 1 capsule or the amount specified. *Of the types of apparatus described herein, use the one specified in the individual monograph. Where the label states that an article is enteric coated and a dissolution or disintegration test does not specifically state that it is to be applied to delayed-release articles and is included in the individual monograph, the procedure and interpretation given for Delayed-Release Dosage Forms are applied, unless otherwise specified in the individual monograph.

FOR DOSAGE FORMS CONTAINING OR COATED WITH GELATIN

If the dosage form containing gelatin does not meet the criteria in the appropriate Acceptance Table (see Interpretation, Immediate-Release Dosage Forms, Extended-Release Dosage Forms, or Delayed-Release Dosage Forms) because of evidence of the presence of cross-linking, the dissolution procedure should be repeated with the addition of enzymes to the medium, as described below, and the dissolution results should be evaluated starting at the first stage of the appropriate Acceptance Table.
It is not necessary to continue testing through the last stage (up to 24 units) when criteria are not met during the first stage testing, and evidence of cross-linking is observed.

Gelatin, in the presence of certain compounds and/or in certain storage conditions, including but not restricted to high humidity and temperature, may present cross-linking. A pellicle may form on the external and/or internal surface of the gelatin capsule shell or on the dosage form that prevents the drug from being released during dissolution testing (see more information in Capsules—Dissolution Testing and Related Quality Attributes (1094)).

[NOTE—All references to a chapter above (1000) are for information purposes only, for use as a helpful resource. These chapters are not mandatory unless explicitly called out for this application.]

**Dissolution Medium with pH ≤4.0**

**Enzyme:** Pepsin, activity determined by the procedure in purified pepsin, in the Reagent Specifications section

**Amount:** A quantity of pepsin that results in an activity of NMT 750,000 Units/L of dissolution medium

**Dissolution Medium with pH >4.0 and <6.8**

**Enzyme:** Papain, activity determined by the Assay test in the monograph for Papain; or bromelain, activity determined by the procedure in bromelain, in the Reagent Specifications section

**Amount:** A quantity of papain that results in an activity of NMT 550,000 Units/L of dissolution medium, or a quantity of bromelain that results in an activity of NMT 30 gelatin-digesting units (GDU)/L of dissolution medium

**Dissolution Medium with pH ≥6.8**

**Enzyme:** Pancreatin, protease activity determined by the procedure in Assay for protease activity (Casein digestive power) in the monograph for Pancreatin

**Amount:** A quantity of pancreatin that results in a protease activity of NMT 2000 Units/L of dissolution medium

### Dissolution Medium Containing Surfactant or Other Ingredients Known to Denature the Enzyme

If the dissolution medium contains surfactant or other ingredients that are known to denature the enzyme used, a pretreatment step in the dissolution testing of the dosage form may be applied. This pretreatment step is done using the specified dissolution medium without the surfactant or the ingredient and with the addition of the appropriate amount of enzyme according to the medium pH. The amount of enzyme added is appropriate to the volume of dissolution medium used in the pretreatment. To achieve the specified medium volume for the final dissolution testing, the pretreatment step may be conducted with a smaller volume of medium without the ingredient such that the final volume is obtained when the ingredient is added at the end of the pretreatment step. All of the other conditions of the test (apparatus, rotation, or flow rate) should remain as described in the method or monograph. Typically, the duration of the pretreatment step is NMT 15 min. The required pretreatment time should be evaluated on a case-by-case basis and should be scientifically justified. This time should be included in the total time of the test. As an example, if the total time of the test is 45 min and 15 min are used in the pretreatment step, the test will continue for 30 min after the addition of the ingredient.

### APPARATUS

**Apparatus 1 (Basket Apparatus)**

The assembly consists of the following: a vessel, which may be covered, and made of glass or other inert, transparent material; a motor; a metallic drive shaft; and a cylindrical basket. The vessel is partially immersed in a suitable water bath of any convenient size or heated by a suitable device, such as a heating jacket. The water bath or heating device permits holding the temperature inside the vessel at 37 ± 0.5° during the test and keeps the bath fluid in constant, smooth motion. No part of the assembly, including the environment in which the assembly is placed, contributes significant motion, agitation, or vibration beyond that due to the smoothly rotating, stirring element. An apparatus that permits observation of the specimen and of the stirring element during the test is preferable. The vessel is cylindrical, with a hemispherical bottom and with one of the following dimensions and capacities: for a nominal, capacity of 1 L, the height is 160–210 mm, and its inside diameter is 98–106 mm; for a nominal capacity of 2 L, the height is 280–300 mm, and its inside diameter is 98–106 mm; and for a nominal capacity of 4 L, the height is 280–300 mm, and its inside diameter is 145–155 mm. Its sides are flanged at the top. A fitted cover may be used to retard evaporation. The shaft is positioned so that its axis is NMT 2 mm at any point from the vertical axis of the vessel and rotates smoothly and without significant wobble that could affect the results. A speed-regulating device is used that allows the shaft rotation speed to be selected and maintained at the specified rate given in the individual monograph, within ±4%. Shaft and basket components of the stirring element are fabricated of stainless steel, type 316, or other inert material, to the specifications shown in Figure 1. A basket having a gold coating of about 0.0001 inch (2.5 µm) thick may be used. A dosage unit is placed in a dry basket at the beginning of each test. The distance between the inside bottom of the vessel and the bottom of the basket is maintained at 25 ± 2 mm during the test.

1 The materials should not sorb, react, or interfere with the specimen being tested.
2 If a cover is used, it provides sufficient openings to allow ready insertion of the thermometer and withdrawal of specimens.
Apparatus 2 (Paddle Apparatus)

Use the assembly from Apparatus 1, except that a paddle formed from a blade and a shaft is used as the stirring element. The shaft is positioned so that its axis is NMT 2 mm from the vertical axis of the vessel at any point and rotates smoothly without significant wobble that could affect the results. The vertical center line of the blade passes through the axis of the shaft so that the bottom of the blade is flush with the bottom of the shaft. The paddle conforms to the specifications shown in Figure 2. The distance of 25 ± 2 mm between the bottom of the blade and the inside bottom of the vessel is maintained during the test. The metallic or suitably inert, rigid blade and shaft compose a single entity. A suitable two-part, detachable design may be used, provided that the assembly remains firmly engaged during the test. The paddle blade and shaft may be coated with a suitable coating so as to make both of them inert. The dosage unit is allowed to sink to the bottom of the vessel before rotation of the blade is started. A small, loose piece of nonreactive material, such as NMT a few turns of wire helix, may be attached to dosage units that would otherwise float. An alternative sinker device is shown in Figure 2a. Other validated sinker devices may be used.
Apparatus 3 (Reciprocating Cylinder)\(^3\)

The assembly consists of a set of cylindrical, flat-bottomed glass vessels; a set of glass reciprocating cylinders; inert fittings (stainless steel type 316 or other suitable material), and screens that are made of suitable nonsorbing and nonreactive material and that are designed to fit the tops and bottoms of the reciprocating cylinders; and a motor and drive assembly to reciprocate the cylinders vertically inside the vessels; if desired, index the reciprocating cylinders horizontally to a different row of vessels. The vessels are partially immersed in a suitable water bath of any convenient size that permits holding the temperature at 37 ± 0.5° during the test. No part of the assembly, including the environment in which the assembly is placed, contributes significant motion, agitation, or vibration beyond that due to the smooth, vertically reciprocating cylinder. A device is used that allows the reciprocation rate to be selected and maintained at the specified dip rate *given in the individual monograph, within ±5%. An apparatus that permits observation of the specimens and reciprocating cylinders is preferable. The vessels are provided with evaporation caps that remain in place for the duration of the test. The components conform to the dimensions shown in Figure 3, unless otherwise specified *in the individual monograph.

\(^3\) Not accepted by the Japanese Pharmacopoeia.
The assembly consists of a reservoir and a pump for the Dissolution medium; a flow-through cell; and a water bath that maintains the Dissolution medium at 37 ± 0.5°. Use the specified cell size as given in the individual monograph.

The pump forces the Dissolution medium upward through the flow-through cell. The pump has a delivery range between 240 and 960 mL/h, with standard flow rates of 4, 8, and 16 mL/min. It must deliver a constant flow (±5% of the nominal flow rate); the flow profile is sinusoidal with a pulsation of 120 ± 10 pulses/min. A pump without pulsation may also be used. Dissolution test procedures using a flow-through cell must be characterized with respect to rate and any pulsation.

The flow-through cell (see Figure 4 and Figure 5), of transparent and inert material, is mounted vertically with a filter system (specified in the individual monograph) that prevents escape of undissolved particles from the top of the cell; standard cell diameters are 12 and 22.6 mm; the bottom cone is usually filled with small glass beads of about 1-mm diameter with one bead of about 5 mm, positioned at the apex to protect the fluid entry tube; and a tablet holder (see Figure 4 and Figure 5) is available for positioning of special dosage forms, e.g., inlay tablets. The cell is immersed in a water bath, and the temperature is maintained at 37 ± 0.5°.
Figure 4. Apparatus 4: large cell for tablets and capsules (top); tablet holder for the large cell (bottom). (All measurements are expressed in mm unless noted otherwise.)
Figure 5. Apparatus 4: small cell for tablets and capsules (top); tablet holder for the small cell (bottom). (All measurements are expressed in mm unless noted otherwise.)

The apparatus uses a clamp mechanism and two O-rings to assemble the cell. The pump is separated from the dissolution unit to shield the latter against any vibrations originating from the pump. The position of the pump should not be on a level higher than the reservoir flasks. Tube connections are as short as possible. Use suitably inert tubing, such as polytef, with about a 1.6-mm inner diameter and chemically inert, flanged-end connections.

**APPARATUS SUITABILITY**

The determination of suitability of a test assembly to perform dissolution testing must include conformance to the dimensions and tolerances of the apparatus as given above. In addition, critical test parameters that have to be monitored periodically during use include volume and temperature of the Dissolution medium, rotation speed (Apparatus 1 and Apparatus 2), dip rate (Apparatus 3), and flow rate of medium (Apparatus 4).

Determine the acceptable performance of the dissolution test assembly periodically.*The suitability for the individual apparatus is demonstrated by the **Performance verification test**.

**USP Reference Standards** (11): **USP Prednisone Tablets RS**

**Performance verification test, Apparatus 1 and Apparatus 2:** Test **USP Prednisone Tablets RS** according to the operating conditions specified. The apparatus is suitable if the results obtained are within the acceptable range stated in the technical data sheet specific to the lot used and the apparatus tested.

**Performance verification test, Apparatus 3:** [To come.]

**Performance verification test, Apparatus 4:** [To come.]
PROCEDURE

Apparatus 1 and Apparatus 2

IMMEDIATE-RELEASE DOSAGE FORMS

Place the stated volume of the Dissolution medium (±1%) in the vessel of the specified apparatus "given in the individual monograph," assemble the apparatus, equilibrate the Dissolution medium to 37 ± 0.5°, and remove the thermometer. Place 1 dosage unit in the apparatus, taking care to exclude air bubbles from the surface of the dosage unit, and immediately operate the apparatus at the specified rate "given in the individual monograph." Within the time interval specified, or at each of the times stated, withdraw a specimen from a zone midway between the surface of the Dissolution medium and the top of the rotating basket or blade, NLT 1 cm from the vessel wall.

[Notes—Where multiple sampling times are specified, replace the aliquots withdrawn for analysis with equal volumes of fresh Dissolution medium at 37° or, where it can be shown that replacement of the medium is not necessary, correct for the volume change in the calculation. Keep the vessel covered for the duration of the test, and verify the temperature of the mixture under test at suitable times.]

Perform the analysis as directed in the individual monograph, using a suitable assay method. Repeat the test with additional dosage form units.

If automated equipment is used for sampling or the apparatus is otherwise modified, verification that the modified apparatus will produce results equivalent to those obtained with the standard apparatus described in this general chapter is necessary.

Dissolution medium: A suitable dissolution medium is used. Use the solvent specified "in the individual monograph." The volume specified refers to measurements made between 20° and 25°. If the Dissolution medium is a buffered solution, adjust the solution so that its pH is within 0.05 unit of the specified pH "given in the individual monograph." [Note—Dissolved gases can cause bubbles to form, which may change the results of the test. If dissolved gases influence the dissolution results, dissolved gases should be removed before testing.]

Time: Where a single time specification is given, the test may be concluded in a shorter period if the requirement for the minimum amount dissolved is met. Specimens are to be withdrawn only at the stated times, within a tolerance of ±2%.

Procedure for a pooled sample for immediate-release dosage forms: Use this procedure where Procedure for a Pooled Sample is specified in the individual monograph. Proceed as directed for Immediate-Release Dosage Forms in Apparatus 1 and Apparatus 2 in the Procedure section. Combine equal volumes of the filtered solutions of the six or twelve individual specimens withdrawn, and use the pooled sample as the test specimen. Determine the average amount of the active ingredient dissolved in the pooled sample.

EXTENDED-RELEASE DOSAGE FORMS

Proceed as directed for Immediate-Release Dosage Forms.

Dissolution medium: Proceed as directed for Immediate-Release Dosage Forms.

Time: The test-time points, generally three, are expressed in hours.

DELAYED-RELEASE DOSAGE FORMS

Use Method A or Method B and the apparatus specified "in the individual monograph." All test times stated are to be observed within a tolerance of ±2%, unless otherwise specified.

Method A Procedure *(unless otherwise directed in the individual monograph), *

ACID STAGE

Place 750 mL of 0.1 N hydrochloric acid in the vessel, and assemble the apparatus. Allow the medium to equilibrate to a temperature of 37 ± 0.5°. Place 1 dosage unit in the apparatus, cover the vessel, and operate the apparatus at the specified rate "given in the monograph."

After 2 h of operation in 0.1 N hydrochloric acid, withdraw an aliquot of the fluid, and proceed immediately as directed in the Buffer Stage.

Perform an analysis of the aliquot using a suitable assay method. *The procedure is specified in the individual monograph.*

BUFFER STAGE

[Note—Complete the operations of adding the buffer and adjusting the pH within 5 min.] With the apparatus operating at the rate specified *in the monograph,* add to the fluid in the vessel 250 mL of 0.20 M tribasic sodium phosphate that has been equilibrated to 37 ± 0.5°. Adjust, if necessary, with 2 N hydrochloric acid or 2 N sodium hydroxide to a pH of 6.8 ± 0.05. Continue to operate the apparatus for 45 min, or for the specified time *given in the individual monograph.* At the end of the

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4 Test specimens are filtered immediately upon sampling unless filtration is demonstrated to be unnecessary. Use an inert filter that does not cause adsorption of the active ingredient or contain extractable substances that would interfere with the analysis.

5 One method of deaeration is as follows: Heat the medium, while stirring gently, to about 41°; immediately filter under vacuum using a filter having a porosity of 0.45 µm or less, with vigorous stirring, and continue stirring under vacuum for about 5 min. Other validated deaeration techniques for removal of dissolved gases may be used.
time period, withdraw an aliquot of the fluid, and perform the analysis using a suitable assay method. The procedure is specified in the individual monograph. The test may be concluded in a shorter time period than that specified for the Buffer Stage if the requirement for the minimum amount dissolved is met at an earlier time.

Method B Procedure *(unless otherwise directed in the individual monograph).*

**ACID STAGE**

Place 1000 mL of 0.1 N hydrochloric acid in the vessel, and assemble the apparatus. Allow the medium to equilibrate to a temperature of 37 ± 0.5°. Place 1 dosage unit in the apparatus, cover the vessel, and operate the apparatus at the rate specified in the monograph. After 2 h of operation in 0.1 N hydrochloric acid, withdraw an aliquot of the fluid, and proceed immediately as directed in the Buffer Stage.

Perform an analysis of the aliquot using a suitable assay method. The procedure is specified in the individual monograph.

**BUFFER STAGE**

[NOTE—For this stage of the procedure, use buffer that has previously been equilibrated to a temperature of 37 ± 0.5°.] Drain the acid from the vessel, and add to the vessel 1000 mL of pH 6.8 phosphate buffer, prepared by mixing 0.1 N hydrochloric acid with 0.20 M tribasic sodium phosphate (3:1) and adjusting, if necessary, with 2 N hydrochloric acid or 2 N sodium hydroxide to a pH of 6.8 ± 0.05. [NOTE—This may also be accomplished by removing from the apparatus the vessel containing the acid, then replacing it with another vessel containing the buffer, and transferring the dosage unit to the vessel containing the buffer.]

Continue to operate the apparatus for 45 min, or for the specified time in the individual monograph. At the end of the time period, withdraw an aliquot of the fluid, and perform the analysis using a suitable assay method. The procedure is specified in the individual monograph. The test may be concluded in a shorter time period than that specified for the Buffer Stage if the requirement for minimum amount dissolved is met at an earlier time.

**Apparatus 3 (Reciprocating Cylinder)**

**IMMEDIATE-RELEASE DOSAGE FORMS**

Place the stated volume of the Dissolution medium in each vessel of the apparatus, assemble the apparatus, equilibrate the Dissolution medium to 37 ± 0.5°, and remove the thermometer. Place 1 dosage form unit in each of the six reciprocating cylinders, taking care to exclude air bubbles from the surface of each dosage unit, and immediately operate the apparatus as specified in the monograph. During the upward and downward strokes, the reciprocating cylinder moves through a total distance of 9.9–10.1 cm. Within the time interval specified, or at each of the times stated, raise the reciprocating cylinders and withdraw a portion of the solution under test from a zone midway between the surface of the Dissolution medium and the bottom of each vessel. Perform the analysis as directed in the individual monograph. If necessary, repeat the test with additional dosage-form units.

**Dissolution medium**: Proceed as directed for Immediate-Release Dosage Forms in Apparatus 1 and Apparatus 2.

**Time**: Proceed as directed for Immediate-Release Dosage Forms in Apparatus 1 and Apparatus 2.

**EXTENDED-RELEASE DOSAGE FORMS**

Proceed as directed for Immediate-Release Dosage Forms in Apparatus 3.

**Dissolution medium**: Proceed as directed for Extended-Release Dosage Forms in Apparatus 1 and Apparatus 2.

**Time**: Proceed as directed for Extended-Release Dosage Forms in Apparatus 1 and Apparatus 2.

**DELAYED-RELEASE DOSAGE FORMS**

Proceed as directed for Delayed-Release Dosage Forms, Method B in Apparatus 1 and Apparatus 2, using one row of vessels for the acid stage media and the following row of vessels for the buffer stage media, and using the volume of medium specified (usually 300 mL).

**Time**: Proceed as directed for Immediate-Release Dosage Forms in Apparatus 1 and Apparatus 2.

**Apparatus 4 (Flow-Through Cell)**

**IMMEDIATE-RELEASE DOSAGE FORMS**

Place the glass beads into the cell specified in the monograph. Place 1 dosage unit on top of the beads or, if specified in the monograph, on a wire carrier. Assemble the filter head, and fix the parts together by means of a suitable clamping device. Introduce by the pump the Dissolution medium warmed to 37 ± 0.5° through the bottom of the cell to obtain the flow rate specified in the individual monograph, and measured with an accuracy of 5%. Collect the eluate by fractions at each of the times stated. Perform the analysis as directed in the individual monograph. Repeat the test with additional dosage form units.

**Dissolution medium**: Proceed as directed for Immediate-Release Dosage Forms in Apparatus 1 and Apparatus 2.

**Time**: Proceed as directed for Immediate-Release Dosage Forms in Apparatus 1 and Apparatus 2.
EXTENDED-RELEASE DOSAGE FORMS

Proceed as directed for Immediate-Release Dosage Forms in Apparatus 4.

Dissolution medium: Proceed as directed for Immediate-Release Dosage Forms in Apparatus 4.

Time: Proceed as directed for Immediate-Release Dosage Forms in Apparatus 4.

DELAYED-RELEASE DOSAGE FORMS

Proceed as directed for Delayed-Release Dosage Forms in Apparatus 1 and Apparatus 2, using the specified media.

Time: Proceed as directed for Delayed-Release Dosage Forms in Apparatus 1 and Apparatus 2.

INTERPRETATION

Immediate-Release Dosage Forms

Unless otherwise specified in the individual monograph, the requirements are met if the quantities of active ingredient dissolved from the dosage units tested conform to Acceptance Table 1. Continue testing through the three stages unless the results conform at either $S_1$ or $S_2$. The quantity, $Q$, is the amount of dissolved active ingredient specified in the individual monograph, expressed as a percentage of the labeled content of the dosage unit; the 5%, 15%, and 25% values in Acceptance Table 1 are percentages of the labeled content so that these values and $Q$ are in the same terms.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Number Tested</th>
<th>Acceptance Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>$S_1$</td>
<td>6</td>
<td>Each unit is NLT $Q + 5%$.</td>
</tr>
<tr>
<td>$S_2$</td>
<td>6</td>
<td>Average of 12 units ($S_1 + S_2$) is $\geq Q$, and no unit is $&lt; Q - 15%$.</td>
</tr>
<tr>
<td>$S_3$</td>
<td>12</td>
<td>Average of 24 units ($S_1 + S_2 + S_3$) is $\geq Q$, NMT 2 units are $&lt; Q - 15%$, and no unit is $&lt; Q - 25%$.</td>
</tr>
</tbody>
</table>

Immediate-Release Dosage Forms Pooled Sample

Unless otherwise specified in the individual monograph, the requirements are met if the quantities of active ingredient dissolved from the pooled sample conform to the accompanying Acceptance Table for a Pooled Sample. Continue testing through the three stages unless the results conform at either $S_1$ or $S_2$. The quantity, $Q_i$, is the amount dissolved at each specified fractional dosing interval. Where more than one range is specified in the individual monograph, the acceptance criteria apply individually to each range.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Number Tested</th>
<th>Acceptance Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>$S_1$</td>
<td>6</td>
<td>Average amount dissolved is NLT $Q + 10%$.</td>
</tr>
<tr>
<td>$S_2$</td>
<td>6</td>
<td>Average amount dissolved ($S_1 + S_2$) is $\geq Q + 5%$.</td>
</tr>
<tr>
<td>$S_3$</td>
<td>12</td>
<td>Average amount dissolved ($S_1 + S_2 + S_3$) is $\geq Q$.</td>
</tr>
</tbody>
</table>

Extended-Release Dosage Forms

Unless otherwise specified in the individual monograph, the requirements are met if the quantities of active ingredient dissolved from the dosage units tested conform to Acceptance Table 2. Continue testing through the three levels unless the results conform at either $L_1$ or $L_2$. Limits on the amounts of active ingredient dissolved are expressed in terms of the percentage of labeled content. The limits embrace each value of $Q_i$, the amount dissolved at each specified fractional dosing interval. Where more than one range is specified in the individual monograph, the acceptance criteria apply individually to each range.

<table>
<thead>
<tr>
<th>Level</th>
<th>Number Tested</th>
<th>Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>$L_1$</td>
<td>6</td>
<td>No individual value lies outside each of the stated ranges, and no individual value is less than the stated amount at the final test time.</td>
</tr>
<tr>
<td>$L_2$</td>
<td>6</td>
<td>The average value of the 12 units ($L_1 + L_2$) lies within each of the stated ranges and is NLT the stated amount at the final test time; none is $&gt;10%$ of labeled content outside each of the stated ranges; and none is $&gt;10%$ of the labeled content below the stated amount at the final test time.</td>
</tr>
</tbody>
</table>
Acceptance Table 2 (continued)

<table>
<thead>
<tr>
<th>Level</th>
<th>Number Tested</th>
<th>Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>$L_2$</td>
<td>12</td>
<td>The average value of the 24 units ($L_1 + L_2 + L_3$) lies within each of the stated ranges and is NLT the stated amount at the final test time; NMT 2 of the 24 units are more than 10% of labeled content outside each of the stated ranges; NMT 2 of the 24 units are &gt;10% of labeled content below the stated amount at the final test time; and none of the units are &gt;20% of labeled content outside each of the stated ranges or &gt;20% of the labeled content below the stated amount at the final test time.</td>
</tr>
</tbody>
</table>

Delayed-Release Dosage Forms

ACID STAGE: Unless otherwise specified *in the individual monograph, the requirements of this portion of the test are met if the quantities, based on the percentage of the labeled content, of active ingredient dissolved from the units tested conform to Acceptance Table 3. Continue testing through all levels unless the results of both the Acid stage and Buffer stage conform at an earlier level.

Acceptance Table 3

<table>
<thead>
<tr>
<th>Level</th>
<th>Number Tested</th>
<th>Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>$A_1$</td>
<td>6</td>
<td>No individual value exceeds 10% dissolved.</td>
</tr>
<tr>
<td>$A_2$</td>
<td>6</td>
<td>Average of the 12 units $(A_1 + A_2)$ is NMT 10% dissolved, and no individual unit is &gt;25% dissolved.</td>
</tr>
<tr>
<td>$A_3$</td>
<td>12</td>
<td>Average of the 24 units $(A_1 + A_2 + A_3)$ is NMT 10% dissolved, and no individual unit is &gt;25% dissolved.</td>
</tr>
</tbody>
</table>

BUFFER STAGE: Unless otherwise specified *in the individual monograph, the requirements are met if the quantities of active ingredient dissolved from the units tested conform to Acceptance Table 4. Continue testing through the three levels unless the results of both stages conform at an earlier level. The value of $Q$ in Acceptance Table 4 is 75% dissolved unless otherwise specified *in the individual monograph. The quantity, $Q$, *specified in the individual monograph, is the total amount of active ingredient dissolved in both the Acid stage and the Buffer stage, expressed as a percentage of the labeled content. The 5%, 15%, and 25% values in Acceptance Table 4 are percentages of the labeled content so that these values and $Q$ are in the same terms.

Acceptance Table 4

<table>
<thead>
<tr>
<th>Level</th>
<th>Number Tested</th>
<th>Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>$B_1$</td>
<td>6</td>
<td>Each unit is NLT $Q + 5%$.</td>
</tr>
<tr>
<td>$B_2$</td>
<td>6</td>
<td>Average of 12 units $(B_1 + B_2)$ is $\geq Q$, and no unit is $&lt; Q - 15%$.</td>
</tr>
<tr>
<td>$B_3$</td>
<td>12</td>
<td>Average of 24 units $(B_1 + B_2 + B_3)$ is $\geq Q$, NMT 2 units are $&lt; Q - 15%$, and no unit is $&lt; Q - 25%$.</td>
</tr>
</tbody>
</table>

Change to read:

The procedure set forth in this chapter determines the amount of volatile matter of any kind that is driven off under the conditions specified, *which is defined as Loss on Drying value.* For substances appearing to contain water as the only volatile constituent, the procedure given in Water Determination (921) is appropriate, and is specified in the individual monograph.

Unless otherwise directed in the individual monograph, conduct the determination of Loss on Drying on a 1- to 2-g test specimen. Mix the substance to be tested and, if it is in the form of large particles, reduce the particle size to about 2 mm by crushing before weighing. Place a glass-stoppered glass-stoppered weighing bottle that has been dried for about 30 min under the same conditions to be employed in the determination and cooled to room temperature in a desiccator. Put the test specimen in the bottle, replace the stopper, and accurately weigh the stopped bottle and the contents. By gentle, sidewise shaking, distribute the test specimen as evenly as practicable to a depth of about 5 mm generally, and NMT 10 mm in the case of low bulk density materials. Place the loaded bottle in the drying chamber, remove the stopper and leave it also in the chamber. Dry the test specimen at the specified temperature and time conditions.

[Note—The Loss on Drying value is a function of both temperature and time. Therefore, these values must be identified and reported. The temperature specified in the monograph is to be regarded as being within the range of ±2° of the stated value.]

When “dry to constant weight” is specified in a monograph, drying shall be continued until two consecutive weighings do not differ by more than 0.50 mg/g of specimen taken, where the second weighing follows an additional hour.
of drying. Upon opening the chamber, reapply the same stopper to the bottle, and allow it to come to room temperature in a desiccator before weighing accurately.

If the substance melts at a lower temperature than that specified for the determination of Loss on Drying, maintain the bottle with its contents for 1–2 h at a temperature 5°–10° below the melting temperature, then dry at the specified temperature. Where capsules are to be tested, use a representative sample mixture, excluding the capsule shell, from not fewer than 4 capsules.

Where tablets are to be tested, use a representative sample mixture from NLT 4 crushed tablets. Where the individual monograph directs that Loss on Drying be determined by thermogravimetric analysis, a suitable balance is to be used (see Balances (41)). Where drying under vacuum over a desiccant is directed in the individual monograph, a vacuum desiccator or a vacuum drying pistol, or other suitable vacuum drying apparatus, is to be used.

Where drying in a desiccator is specified, exercise particular care to ensure that the desiccant is fully effective. Where drying in a capillary-stoppered bottle under vacuum is directed in the individual monograph, use a bottle or tube fitted with a stopper having a 225 ± 25-µm diameter capillary, and maintain the heating chamber at a pressure of 5 mm or less of mercury. At the end of the heating period, admit dry air to the heating chamber, remove the bottle, and with the capillary stopper still in place allow it to cool to room temperature in a desiccator before weighing.

〈736〉 MASS SPECTROMETRY

INTRODUCTION

Mass spectrometry (MS) is an analytical technique based on the measurement of the mass-to-charge ratio of ionic species related to the analyte under investigation. MS can be used to determine the molecular mass and elemental composition of an analyte as well as provide an in-depth structural elucidation of the analyte.

In addition to being recognized as a powerful structure-elucidation tool, MS is also extensively used for quantitative measurements. For additional information, see general information chapter Applications of Mass Spectrometry (1736), which provides a detailed discussion of MS.

Currently available MS instrumentation offers a wide range of capabilities for qualitative and quantitative analysis, which results in a wide range of potential MS experimental approaches for a given measurement need. Because of the diversity of approaches, this chapter does not present specific procedures but instead provides experimental and system suitability information for MS procedures.

QUALITATIVE ANALYSIS

MS is a sensitive and highly specific technique for the identification of analytes. Identification or verification of structure (i.e., comparison against an authentic standard) by MS is particularly powerful when used in conjunction with a separation technique such as gas chromatography (GC) or high-performance liquid chromatography (HPLC). Additional degrees of specificity can be obtained by the use of tandem mass spectrometry (MS/MS) or high-resolution mass spectrometry (HRMS). Also, tighter tolerances on mass accuracy are also afforded using high-resolution mass spectrometry (HRMS).

Experimental Parameters

The following MS experimental parameters should be defined for a qualitative (e.g., identification) procedure.

MASS RESOLUTION

Unit mass resolution is sufficient for most identification tests. When higher resolution is required, the resolution is specified in the procedure, and demonstration of adequate resolution is included in the system suitability tests for the procedure.

MASS ACCURACY

A mass accuracy or agreement of ±0.50 mass units for singly charged ions from a known standard should be sufficient for most applications. A mass accuracy or agreement of ±0.05% from a standard should be sufficient for the identification of large molecules (above 2000 m/z) when multiply charged ions are employed for the identification test.

When higher mass accuracy is required, the required mass accuracy is specified in the procedure. A demonstration of the mass accuracy then is included in the system suitability tests for the procedure or as part of the instrument’s established performance qualification (PQ) procedure discussed later in this chapter.

1 Available as an “antibiotic moisture content flask” from Kimble Chase Life Science and Research Products, 1022 Spruce St., Vineland, NJ 08360.
MASS RANGE

The mass range to be scanned is also presented in the procedure. The mass range must encompass all ions used as part of the identification confirmation.

System Suitability

The system suitability for the MS procedure should include demonstration of adequate performance for the following experimental attributes.

MASS RESOLUTION

A demonstration of the appropriate resolution is included in the system suitability tests for a procedure. The performance test in the instrument’s established PQ, executed daily or prior to the time of use, may suffice. When resolutions greater than unit mass are required for a procedure, the system suitability test includes demonstration of adequate resolution along with acceptance criteria.

MASS ACCURACY

A demonstration of the mass accuracy is included in the system suitability tests for the procedure or is part of the instrument’s established PQ procedure. A mass accuracy or agreement of ±0.50 mass units for singly charged ions from a known standard should be sufficient for most applications. If higher mass accuracy is required for a given procedure, the appropriate acceptance criteria are specified.

Interpretation Directions

An identification or verification experiment involves comparison of the compound of interest to an authentic standard (e.g., a USP Reference Standard). For this form of identification or verification, the standard and the sample are run under identical conditions and should have results that are within acceptable experimental error for the procedure. Applications of MS for identification purposes in a compendial monograph may incorporate the mass spectral data alone or may combine this spectral identification with the chromatographic retention time if more specificity is dictated by the particular monograph identification needs.

The procedure should also provide specific instructions that define a successful mass spectral match. If the procedure represents the only spectroscopic identification test or if no other identification tests (e.g., peptide mapping or amino acid analysis) provide structural information, the spectrum of the standard should closely resemble that of the sample and a minimum of three structurally relevant ions, preferably one of which is an ion representing the molecular mass of the analyte, should be used for the comparison. In the case where only the ion representing the intact molecule is produced, the accurate mass or MS/MS spectrum of the molecular ion may be used to strengthen the identification. If other structurally relevant identification tests are also conducted, the comparison can be conducted with fewer ions as long as one of the ions represents the molecular mass of the analyte. For example, the MS identification test for a protein may examine only an ion that represents the molecular mass if several other tests are conducted to confirm the protein structure.

QUANTITATIVE ANALYSIS

The sensitivity and specificity of MS also make it a suitable analytical tool for the quantification of analytes. Quantification is particularly powerful when used in conjunction with a separation technique such as GC or HPLC. Further degrees of specificity can be obtained by the use of MS/MS or HRMS.

Experimental Parameters

The following experimental parameters should be defined within a quantitative (e.g., identification) MS procedure.

MASS RESOLUTION

Unit mass resolution is sufficient for most quantitative tests. When higher resolution is required, the resolution is specified in the procedure, and demonstration of the mass accuracy is included in the system suitability tests for the procedure.

MASS ACCURACY

The mass accuracies listed in the previous qualitative section should be sufficient for most quantitative applications. When higher mass accuracy is required, it is specified in the procedure. A demonstration of the mass accuracy is included in the system suitability tests for the procedure or as part of the instrument’s established PQ procedure.

MASS SELECTION

The masses to be monitored (e.g., mass range, individual masses, or MS/MS transitions) are presented in the procedure.
System Suitability

The system suitability for the MS procedure should include demonstration of adequate performance for the following experimental attributes, as appropriate for the procedure.

MASS RESOLUTION

A demonstration of the appropriate resolution is included in the system suitability tests for the procedure or is part of the instrument’s established PQ procedure. When resolutions greater than unit mass are required for a procedure, the system suitability test includes demonstration of adequate resolution along with acceptance criteria.

MASS ACCURACY

A demonstration of the mass accuracy is included in the system suitability tests for the procedure or is part of the instrument’s established PQ procedure. A mass accuracy or agreement of ±0.50 mass units for singly charged ions from a known standard should be sufficient for most applications. If higher mass accuracy is required for a given procedure, the appropriate acceptance criteria are specified.

PRECISION

The system suitability includes a demonstration of adequate precision. See Table 1 for maximum limits for precision. Typically, system suitability limits are set to tighter limits than those employed to ensure adequate precision for the validation experiments. (See also the section on Validation and Verification of Mass Spectrometry Analytical Procedures.)

LINEARITY

The system suitability includes a demonstration of adequate linearity. See Table 1 for appropriate linearity limits. (See also the section on Validation and Verification of Mass Spectrometry Analytical Procedures.)

ACCURACY

In certain situations, quality control (or check) samples may also be appropriate for inclusion in the procedure to ensure the quality of the measurement. Typically, these quality control samples are of known analyte concentration and are prepared identically to the test samples. If used, quality control (or check) samples are also prepared as a verification of time-of-application method accuracy. The procedure specifies the number or analysis order of quality control (or check) samples needed. Acceptance criteria for calibration and quality control (or check) sample results should be aligned with the validation requirements as required by the application type (i.e., Category I or II) as outlined in Table 1.

QUANTITATION LIMIT

In certain applications (e.g., limits tests), it may be necessary to include demonstration of the ability to detect the analyte at a prescribed level. For these applications, the procedure specifies the limit and success criteria (e.g., signal-to-noise ratio).

QUALIFICATION OF MASS SPECTROMETRY INSTRUMENTS

Qualification of an MS instrument can be divided into three elements: installation qualification (IQ), operational qualification (OQ), and PQ. For additional information, see Analytical Instrument Qualification (1058), which may be a helpful, but not mandatory resource.

Installation Qualification

IQ provides evidence that the hardware and software are installed to accommodate safe and effective use of the instrument at the desired location.

Operational Qualification

In OQ, an instrument’s performance is characterized using standards to verify that the system operates within target specifications. The purpose of OQ is to demonstrate that instrument performance is suitable for a given application. Because so many different approaches are available for measuring MS spectra, OQ using standards with known spectral properties is recommended. Because of the diversity of MS instrumentation, interfaces, and experimental approaches, MS instruments should be qualified against target specifications for the intended application, not simply the specifications supplied by the manufacturer.

Performance Qualification

PQ helps to determine that the instrument is capable of meeting the user’s requirements for all critical-to-quality measures. PQ documentation should describe the following:
• the definition of the specific performance criteria and detailed test procedures, including test samples and instrument parameters;
• the elements that will be measured to evaluate the criteria and the predefined specifications;
• the test interval, which may be daily or time-of-use measurements;
• the use of bracketing samples or groups of samples; and
• corrective actions that will be implemented if the spectrometer does not pass the specifications.

Periodic PQ should include a subset of the OQ tests to ensure that the instrument as supplied is performing at a level that produces data that are suitable for their intended use. Depending on typical use, the specifications for PQ may be higher or lower than the manufacturer's installation specifications. Method-specific PQ tests, also known as system suitability tests, may be used in lieu of PQ requirements for validated procedures.

Because of the diversity of MS instrumental configurations and experimental designs, a standard sample or experiment for all PQ assessments may not be available. Thus, method-specific PQ tests or system suitability tests often are needed. The PQ experimental design should be sufficiently robust to ensure proper instrument performance for the intended application, including the specifications associated with the measurement. At minimum, PQ experiments should include the following.

• For qualitative applications, the PQ experiment includes a check of the mass accuracy of the instrumentation. A mass accuracy or agreement of ±0.50 mass units for singly charged ions from a known standard should be sufficient for most applications.

• For quantitative applications, the PQ experiment includes checks of mass accuracy and precision. A mass accuracy or agreement of ±0.50 mass units for singly charged ions from a known standard should be sufficient for most applications. The success criteria for precision is established via consideration of the instrument and method capability, and provides sufficient controls relative to the specification for the measurement in question.

Characterizing Instrument Performance

Specific procedures, acceptance criteria, and time intervals for characterizing MS spectrometer performance depend on the instrument and its intended applications. Many MS applications use previously validated experiments that relation MS spectra to a chemical property of interest. Analysts typically demonstrate stable instrument performance over extended periods of time. This practice provides some assurance that reliable measurements can be taken from sample spectra using previously validated MS experiments.

VALIDATION AND VERIFICATION OF MASS SPECTROMETRY ANALYTICAL PROCEDURES

Validation is required only when an MS procedure is an alternative to the official procedure for testing an official article. The objective of validating an MS procedure is to demonstrate that the measurement is suitable for its intended purpose, including quantitative determination of the main component in a drug substance or a drug product (Category I assays), quantitative determination of impurities (Category II), and identification tests (Category IV). [NOTE—For additional information on the different category definitions, see Validation of Compendial Procedures (1225).] Depending on the category of the test, analytical procedure validation requires the testing of linearity, range, accuracy, specificity, precision, quantitation limit, and robustness. These analytical performance characteristics apply to externally standardized methods and to the method of standard additions.

Chapter (1225) provides definitions and general guidance about analytical procedures validation without indicating specific validation criteria for each characteristic. The intention of the following sections is to provide the user with specific validation criteria that represent the minimum expectations for this technology. For each particular application, tighter criteria may be needed in order to demonstrate suitability for the intended use.

Measurement Categories for Mass Spectrometry Analytical Procedures

The required validation performance characteristics of an MS analytical procedure, assuming the typical Category I USP specifications of 98.0%–102.0% for drug substances and 95.0%–105.0% for drug products, are listed in Table 1. The actual validation performance characteristics would be dependent upon the specifications in place and should provide sufficient evidence that the measurement capability is sufficient for those specifications. A procedure validation protocol must specify the required validation experiments and validation criteria. These criteria are determined according to the intended purpose of the analytical procedure.

<table>
<thead>
<tr>
<th>Analytical Performance Characteristics</th>
<th>Category I</th>
<th>Category II Quantitative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specificity</td>
<td>Ensured by use of a reference standard when possible and demonstrable lack of interference from other components</td>
<td></td>
</tr>
<tr>
<td>Linearity</td>
<td>Correlation coefficient (R) NLT 0.995</td>
<td>Correlation coefficient (R) NLT 0.99</td>
</tr>
</tbody>
</table>
Table 1. Analytical Measurement Requirements (continued)

<table>
<thead>
<tr>
<th>Analytical Performance Characteristics</th>
<th>Category I</th>
<th>Category II Quantitative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Range</td>
<td>For 100.0% centered acceptance criteria: 80.0%–120.0%. For noncentered acceptance criteria: 10.0% below the lower limit to 10.0% above the upper limit. For content uniformity: 70.0%–130.0%</td>
<td>50%–120%</td>
</tr>
<tr>
<td>Accuracy</td>
<td>98.0%–102.0% (drug substance) 95.0%–105.0% (drug product)</td>
<td>80.0%–120.0%</td>
</tr>
<tr>
<td>Repeatability</td>
<td>NMT 1.0% (drug substance) NMT 2.0% (drug product)</td>
<td>NMT 20.0%</td>
</tr>
<tr>
<td>Intermediate precision</td>
<td>NMT 1.0% (drug substance) NMT 3.0% (drug product)</td>
<td>NMT 25.0%</td>
</tr>
<tr>
<td>Quantitation limit</td>
<td>—</td>
<td>The analytical procedure should be capable of determining the analyte precisely and accurately at a level equivalent to 50% of the specification.</td>
</tr>
<tr>
<td>Robustness</td>
<td>The reliability of an analytical measurement should be demonstrated by deliberate changes to experimental parameters.</td>
<td></td>
</tr>
</tbody>
</table>

Analytical Procedure Validation

The objective of analytical procedure validation is to demonstrate that the analytical procedure is suitable for its intended purpose by conducting experiments and obtaining results that meet predefined acceptance criteria. MS analytical procedures can include quantitative tests for major component and impurities content, limit tests for the presence of impurities, quantification of a component in a product or formulation, or identification tests.

VALIDATION PARAMETERS

Performance characteristics that demonstrate the suitability of an analytical procedure are similar to those required for any analytical procedure. For additional information on the applicable general principles, see (1225). Specific acceptance criteria for each validation parameter must be consistent with the intended use of the analytical procedure.

The performance characteristics that are required as part of a validation for each of the analytical procedure categories are given in Table 1.

Specificity: The purpose of a specificity test is to demonstrate that measurements of the intended analyte signals are free of interference from components and impurities in the test material. Specificity tests can be conducted to compare spectra of components and impurities that are known from synthetic processes, formulations, and test preparations. Specificity is also to be demonstrated for any materials added as part of the procedure (e.g., specificity versus isotope-labeled internal standards). For an identification MS analytical procedure (Category I and II), validation experiments may include multidimensional MS experiments to validate correct assignments of an ion’s structure or origin.

Linearity: A linear relationship is exhibited between the analyte concentration and instrument response. This is demonstrated by measuring analyte responses from NLT five standard solutions at concentrations that encompass the anticipated concentration range of analyte(s) in the test solution. For Category I, standard solutions can be prepared from reference materials in appropriate solvents. For Category II (MS analytical procedures that are used to quantitate impurities), linearity samples are prepared by spiking suitable test samples that contain low amounts of analyte or by spiking matrix samples at concentrations of the expected range. The standard curve then is constructed using appropriate statistical analytical procedures such as a least-squares regression. The correlation coefficient ($R$), y-intercept, slope of the regression line, and residual root mean square are then determined. Absolute values determined for these factors are appropriate for the procedure being validated.

Range: The range between the low and high concentrations of analyte is given by the quantitative MS analytical procedure. This typically is based on test article specifications in the USP monograph. It is the range within which the analytical procedure can demonstrate an acceptable degree of linearity, accuracy, and precision and can be obtained from an evaluation of that analytical procedure. Recommended ranges for various MS analytical procedures are as follows.

- For Category I—assay of a drug substance (or a finished product): 80%–120% of the test concentration;
- For Category I—content uniformity: a minimum of 70%–130% of the test concentration;
- For Category II—determination of an impurity: 50%–120% of the acceptance criteria.

Accuracy: The accuracy of a quantitative MS analytical procedure is determined across the required analytical range. Typically, three levels of concentrations are evaluated using triplicate preparations at each level.

Preparation of accuracy samples: For drug substance assays (Category I), accuracy is determined by analyzing a reference standard of known purity. For drug product assays (Category I), a composite sample of reference standard and other components in a pharmaceutical finished product should be used for analytical procedure validation. The assay results are compared to the theoretical value of the reference standard to estimate errors or percent recovery. For the quantitation of impurities (Category II), the accuracy of the analytical procedure can be determined by conducting studies with drug substances or products spiked with known concentrations of the analyte under test.
Assay results from the analytical procedure being validated may be compared to those of an established alternative analytical procedure.

**Precision:**

- **Repeatability:** The analytical procedure is assessed by measuring the concentrations of three replicates of separate standard solutions at three different concentrations that encompass the analytical range. Alternatively, the concentrations of six separate standard solutions at 100% of the test concentration can be measured. The relative standard deviation from the replicate measurements is then evaluated to determine if the solutions meet the acceptance criteria.

- **Intermediate precision:** The effect of random events on the analytical precision of the analytical procedure is to be established. Typical variables include performing the analysis on different days, using different instruments that are suitable as specified in the analytical procedure, or having the analytical procedure performed by two or more analysts.

**Quantitation limit:** The quantitation limit is validated by measuring six replicates of test samples spiked with analyte at 50% of specification. From these replicates, analysts are then able to determine accuracy and precision. Examples of specifications for Category II quantitative determinations are that the measured concentration is within 70%–130% of the spike concentration and the relative standard deviation is NMT 15%.

**Robustness:** The reliability of an analytical measurement is demonstrated with deliberate changes to critical experimental parameters. These can include measuring the stability of the analyte under specified storage, chromatographic, or ionization conditions.

### Analytical Procedure Verification

U.S. Current Good Manufacturing Practices regulations [21 CFR 211.194(a)(2)] indicate that users of analytical procedures described in USP–NF do not need to validate procedures that are provided in a monograph. Instead, they must simply verify the suitability of the procedures under actual conditions of use.

The objective of an MS procedure verification is to demonstrate that the procedure as prescribed in a specific monograph can be executed by the user with suitable accuracy, specificity, and precision using the instruments, analysts, and sample matrices available. According to the general information chapter Verification of Compendial Procedures (1226), if the verification of the compendial procedure by following the monograph is not successful, the procedure may not be suitable for use with the article under test. It may be necessary to develop and validate an alternative procedure as allowed in General Notices, 6.30 Alternative and Harmonized Methods and Procedures.

Verification of a compendial MS procedure includes at minimum the execution of the validation parameters for specificity, accuracy, precision, and limit of quantitation, when appropriate, as indicated in the Validation and Verification of Mass Spectrometry Analytical Procedures section in this chapter.

### 〈741〉 MELTING RANGE OR TEMPERATURE

The terms melting range, melting point, or melting temperature are all used in pharmacopeial contexts. Most substances exhibit a melting transition, spanning the temperatures at which the first detectable change of phase or liquid phase is detected to the temperature at which no solid phase is apparent. The transition may appear instantaneous for a highly pure material, but usually a range is observed from the beginning to the end of the process. Factors influencing this transition include the sample size, the particle size, the efficiency of heat diffusion within the sample, and the heating rate, among other variables, that are controlled by procedure instructions.

For pharmacopeial purposes, the temperatures of the beginning (onset temperature) and end of transition (clear temperature) represent the melting range, except as defined otherwise for Procedure for Class II and Procedure for Class III below. The terms melting point and melting temperature are considered to be equivalent.

Substances which melt with no decomposition or chemical change are known to melt congruently. In these cases, the melting point is taken to be the end of the melting range, i.e., the temperature at which no solid phase is apparent. In some articles, the melting process is accompanied by simultaneous decomposition, which is visually evidenced as a side event like darkening of the material, charring, bubbling, or other incident. These transitions are known to be non-congruent. The visual impact of this side reaction frequently obscures the end of the melting process, which may be impossible to accurately determine. In those circumstances, only the beginning of the melting can be accurately established, and it is to be reported as the melting point.

Since there may be a thermal lag between the heating medium and the sample within the capillary tube, in order to achieve consistency and repeatability, it is important to perform the melting determination at a heating rate, also referred to as ramp rate, of $1^\circ$/min.

The accuracy of the apparatus to be used as described below should be checked at suitable intervals by the use of one or more of the available USP Melting Point Standards, preferably those that melt nearest the melting temperatures of the compounds being tested (see USP Reference Standards (11)). The USP Melting Point Standards are intended to check the accuracy of the device and may be suitable to calibrate.

Eight procedures for the determination of melting range or temperature are given herein, varying in accordance with the nature of the substance. When no class is designated in the monograph, use the Procedure for Class I for crystalline or amorphous substances and the Procedure for Class II for waxy substances.

The procedure known as the mixed-melting point determination, whereby the melting range or temperature of a solid under test is compared with that of an intimate mixture of equal parts of the solid and an authentic specimen of it, e.g., the corresponding USP Reference Standard, if available, may be used as a confirmatory identification test. Agreement of the observations on the original and the mixture constitutes reliable evidence of chemical identity.
**APPARATUS**

Apparatus with cameras or other computerized equipment to improve accuracy, sensitivity, or precision may be used provided that the apparatus is properly qualified.

**Apparatus I**

An example of a suitable melting range **Apparatus I** consists of a glass container for a bath of transparent fluid, a suitable stirring device, an accurate thermometer, and a controlled source of heat. The bath fluid is selected with a view to the temperature required, but light paraffin is used generally and certain liquid silicones are well adapted to the higher temperature ranges. The fluid is deep enough to permit immersion of the thermometer to its specified immersion depth so that the bulb is still about 2 cm above the bottom of the bath. The heat may be supplied by an open flame or electrically. The capillary tube is about 10 cm long and 0.8–1.2 mm in internal diameter with walls 0.2–0.3 mm in thickness.

**Apparatus II**

An instrument may be used in the **Procedure for Class I**, **Procedure for Class Ia**, and **Procedure for Class Ib**. An example of a suitable melting range **Apparatus II** consists of a block of metal that may be heated at a controlled rate, with its temperature being monitored by a sensor. The block accommodates the capillary tube containing the test substance and permits monitoring of the melting process, typically by means of a beam of light and a detector. The detector signal may be processed by a microcomputer to determine and display the melting point or range, or the detector signal may be plotted to allow visual estimation of the melting point or range. Some approaches broadly used by automated systems employ optical methods such as light absorption or bulk reflection.

**PROCEDURES**

**Procedure for Class I, Apparatus I**

Reduce the substance under test to a very fine powder, and, unless otherwise directed, render it anhydrous when it contains water of hydration by drying it at the temperature specified in the monograph, or, when the substance contains no water of hydration, dry it over a suitable desiccant for NLT 16 h (or at the conditions stated in **Loss on Drying** (731), if appropriate).

Charge a capillary glass tube, one end of which is sealed, with a sufficient amount of the dry powder to form a column in the bottom of the tube to a nominal height of 3 mm high when packed down as closely as possible by moderate tapping on a solid surface. Due to the instrument design, alternative sample sizes may be used as instructed by the instrument manufacturer.

Heat the bath until the temperature is about 10° below the expected melting point. Remove the thermometer, and quickly attach the capillary tube to the thermometer by wetting both with a drop of the liquid of the bath or otherwise, and adjust its height so that the material in the capillary is level with the thermometer bulb. Replace the thermometer, and continue heating, with constant stirring, sufficiently to achieve the ramp rate of about 3°/min. When the temperature is about 3° below the lower limit of the expected melting range, reduce the heating so that the ramp rate of about 1°/min is achieved. Continue heating until melting is complete.

The temperature at which the column of the substance under test is observed to collapse definitely against the side of the tube at any point indicates the beginning of melting, and the temperature at which the test substance becomes liquid throughout corresponds to the end of melting or the melting point. The two temperatures fall within the limits of the melting range. If melting occurs with decomposition, the melting temperature corresponding to the beginning of the melting (melting point) is within the range specified.

**Procedure for Class Ia, Apparatus I**

Prepare the test substance and charge the capillary as directed in **Procedure for Class I, Apparatus I**. Heat the bath until the temperature is about 10° below the expected melting point and is rising at a rate of about 1°/min. Insert the capillary as directed in **Procedure for Class I, Apparatus I** when the temperature is about 5° below the lower limit of the expected melting range, and continue heating until melting is complete. Record the melting range as directed in **Procedure for Class I, Apparatus I**.

**Procedure for Class Ib, Apparatus I**

Place the test substance in a closed container and cool to 10°, or lower, for at least 2 h. Without powdering, charge the cooled material into the capillary tube as directed in **Procedure for Class I, Apparatus I**, then immediately place the charged tube in a vacuum desiccator and dry at a pressure not exceeding 20 mm of mercury for 3 h. Immediately upon removal from the desiccator, fire-seal the open end of the tube, and as soon as practicable proceed with the determination of the melting range as follows. Heat the bath until the temperature is about 10° below the expected melting range, then introduce the charged tube, and heat at a rate of rise of about 1°/min until melting is complete. Record the melting range as directed in **Procedure for Class I, Apparatus I**.

If the particle size of the material is too large for the capillary, precool the test substance as directed above, then with as little pressure as possible gently crush the particles to fit the capillary, and immediately charge the tube.
Procedure for Class I, Apparatus II

Prepare the substance under test and charge the capillary tube as directed in Procedure for Class I, Apparatus I. Operate the apparatus according to the manufacturer’s instructions. Heat the block until the temperature is about 10° below the expected melting point. Insert the capillary tube into the heating block, and continue heating at a rate of temperature increase of about 1°/min until melting is complete. The temperature at which the detector signal first leaves its initial value indicates the beginning of melting, and the temperature at which the detector signal reaches its final value corresponds to the end of melting, or the melting point. The two temperatures fall within the limits of the melting range. If melting occurs with decomposition, the melting temperature corresponding to the beginning of the melting (melting point) is within the range specified.

Procedure for Class Ia, Apparatus II

Prepare the test substance and charge the capillary as directed in Procedure for Class I, Apparatus I. Operate the apparatus according to the manufacturer’s instructions. Heat the block until the temperature is about 5° below the expected melting point and is rising at a rate of about 1°/min. Insert the capillary as directed in Procedure for Class I, Apparatus I, and continue heating until melting is complete. Record the melting range as directed in Procedure for Class I, Apparatus I. If melting occurs with decomposition, the melting temperature corresponding to the beginning of the melting (melting point) is within the range specified.

Procedure for Class Ib, Apparatus II

Place the test substance in a closed container and cool to 10°, or lower, for at least 2 h. Without previous powdering, charge the cooled material into the capillary tube as directed in Procedure for Class I, Apparatus I, then immediately place the charged tube in a vacuum desiccator, and dry at a pressure not exceeding 20 mm of mercury for 3 h. Immediately upon removal from the desiccator, fire-seal the open end of the tube, and as soon as practicable proceed with the determination of the melting range as follows. Operate the apparatus according to the manufacturer’s instructions. Heat the block until the temperature is about 10° below the expected melting range, then introduce the charged tube, and heat at a rate of rise of about 1°/min until melting is complete. If the particle size of the material is too large for the capillary, precool the test substance as directed above, then with as little pressure as possible gently crush the particles to fit the capillary, and immediately charge the tube.

Procedure for Class II

Carefully melt the material to be tested at as low a temperature as possible, and draw it into a capillary tube, which is left open at both ends, to a depth of about 10 mm. Cool the charged tube at 10°, or lower, for 24 h, or in contact with ice for at least 2 h. Then attach the tube to the thermometer by suitable means, adjust it in a water bath so that the upper edge of the material is 10 mm below the water level, and heat as directed in Procedure for Class I, Apparatus I except, within 5° of the expected melting temperature, to regulate the rate of rise of temperature of about 1.0°/min. The temperature at which the material is observed to rise in the capillary tube is the melting temperature.

Procedure for Class III

While stirring, melt a quantity of the test substance slowly until it reaches a temperature of 90°–92°. Remove the source of the heat, and allow the molten substance to cool to a temperature of 8°–10° above the expected melting point. Chill the bulb of a suitable thermometer to 5°, wipe it dry, and while it is still cold dip it into the molten substance so that approximately the lower half of the bulb is submerged. Withdraw it immediately, and hold it vertically away from the heat until the wax surface dulls, then dip it for 5 min into a water bath having a temperature NMT 16°. Fix the thermometer securely in a test tube so that the lower point is 15 mm above the bottom of the test tube. Suspend the test tube in a water bath adjusted to about 16°, and raise the temperature of the bath at the rate of about 2°/min to 30°, then change to a rate of about 1°/min, and note the temperature at which the first drop of melted substance leaves the thermometer. Repeat the determination twice on a freshly melted portion of the test substance. If the variation of three determinations is less than 1°, take the average of the three as the melting point. If the variation of three determinations is 1° or greater than 1°, make two additional determinations and take the average of the five.

〈781〉 OPTICAL ROTATION

INTRODUCTION

Many pharmaceutical substances are optically active in the sense that they rotate an incident plane of polarized light so that the transmitted light emerges at a measurable angle to the plane of the incident light. This property is characteristic of some crystals and of many pharmaceutical liquids or solutions of solids. Where the property is possessed by a liquid or by a solute in solution, it is generally the result of the presence of one or more asymmetric centers, usually a carbon atom with four different substituents. The number of optical isomers is 2ⁿ, where n is the number of asymmetric centers. Polarimetry, the measurement
of optical rotation, of a pharmaceutical article may be the only convenient means for distinguishing optically active isomers from each other and thus is an important criterion of identity and purity. Substances that may show opt rotatory properties are “chiral”. Those that rotate light in a clockwise direction as viewed toward the light source are “dextrorotatory”, or “(+)-optical isomers”, and those that rotate light in a counterclockwise direction are called “levorotatory” or “(−)-optical isomers”.

The physicochemical properties of nonsuperimposable chiral substances rotating plane-polarized light in opposite directions to the same extent, “enantiomers”, are identical, except for this property and in their reactions with other chiral substances. Enantiomers often exhibit profound differences in pharmacology and toxicology, owing to the fact that biological receptors and enzymes themselves are chiral. Many articles from natural sources, such as amino acids, proteins, alkaloids, antibiotics, glycosides, and sugars, exist as chiral compounds. Synthesis of such compounds from nonchiral materials usually results in equal amounts of the enantiomers, i.e., “racemates”. Racemates have a null optical rotation, and their physical properties may differ from those of the component enantiomers. Use of stereoselective or stereospecific synthetic methods or separation of racemic mixtures can be used to obtain individual optical isomers.

Measurement of optical rotation is performed using a polarimeter. The general equation used in polarimetry is:

\[
\alpha = \frac{100a}{lc}
\]

Thus, \( \alpha \) is 100 times the measured value, in degrees (°), for a solution containing 1 g in 100 mL, measured in a cell having a path length of 1.0 dm under defined conditions of incident wavelength of light and temperature. For some Pharmacopeial articles, especially liquids such as essential oils, the optical rotation requirement is expressed in terms of the observed rotation, \( a \), measured under conditions defined in the monograph.

Historically, polarimetry was performed using an instrument where the extent of optical rotation is estimated by visual matching of the intensity of split fields. For this reason, the D-line of the sodium lamp at the visible wavelength of 589 nm was most often employed. Specific rotation determined at the D-line is expressed by the symbol:

\[
[\alpha]_D^{25}
\]

Much of the data available are expressed in this form. Use of lower wavelengths, such as those available with the mercury lamp lines isolated by means of filters of maximum transmittance at approximately 546, 436, 405, 365, and 325 nm in a photoelectric polarimeter, has been found to provide advantages in sensitivity with a consequent reduction in the concentration of the test compound. In general, the observed optical rotation at 436 nm is about double, and at 365 nm, about 3 times that at 589 nm. Reduction in the concentration of the solute required for measurement may sometimes be accomplished by conversion of the substance under test to one that has a significantly higher optical rotation. Optical rotation is also affected by the solvent used for the measurement, and this is always specified.

It is now common practice to use other light sources, such as xenon or tungsten halogen, with appropriate filters, because these may offer advantages of cost, long life, and broad wavelength emission range, over traditional light sources.

**PROCEDURES**

**Specific Rotation**

The reference “Optical Rotation (781S), Procedures, Specific Rotation” in a monograph signifies that specific rotation is to be calculated from observed optical rotations in the Test solution or Sample solution obtained as directed therein. Unless otherwise directed, measurements of optical rotation are made in a 1.0-dm tube at 589 nm at 25°C. Where a photoelectric polarimeter is used, a single measurement, corrected for the solvent blank, is made. Where a visual polarimeter is employed, the average of no fewer than five determinations, corrected for the reading of the same tube with a solvent blank, is used. Temperature, which applies to the solution or the liquid under test, should be maintained within 0.5°C of the stated value. Use the same cell for sample and blank. Maintain the same angular orientation of the cell in each reading. Place the cell so that the light passes through it in the same direction each time. Unless otherwise specified, specific rotation is calculated on the dried basis where Loss on Drying is specified in the monograph, or on the anhydrous basis where Water Determination is specified.

1 Suitable calibrators are available from the Office of Standard Reference Materials, National Institute of Standards and Technology (NIST), Gaithersburg, MD 20899, as current lots of Standard Reference Materials, Dextrate and Sucrose. Alternatively, calibration may be checked using a Polarization Reference Standard, which consists of a plate of quartz mounted in a holder perpendicular to the light path. Acceptable periodic calibrations may be made by a National Metrology Institute (NMI) that is signatory to the International Committee of Weights and Measures (CIPM) Mutual Recognition Arrangement (MRA) or an ISO/IEC 17025 accredited laboratory. For calibration where the accreditation body is a signatory to the International Laboratory Accreditation Cooperation (ILAC) MRA.

2 All references of wavelengths are in vacuum. The sodium D-line is 589.44 in vacuum and 589.3 in air.
Optical rotation of solutions should be determined within 30 min of preparation. In the case of substances known to undergo racemization or mutarotation, care should be taken to standardize the time between adding the solute to the solvent and introduction of the solution into the polarimeter tube.

Angular Rotation

The reference “Optical Rotation (781A), Procedures, Angular Rotation” in a monograph signifies, unless otherwise directed, that the optical rotation of the neat liquid is measured in a 1.0-dm tube at 589 nm at 25°, corrected for the reading of the dry, empty tube.2

〈785〉 OSMOLALITY AND OSMOLARITY

INTRODUCTION

Osmotic pressure plays a critical role in all biological processes that involve diffusion of solutes or transfer of fluids through membranes. Osmosis occurs when solvent but not solute molecules cross a semipermeable membrane from regions of lower to higher concentrations to produce equilibrium. The knowledge of osmotic pressures is important for practitioners in determining whether a parenteral solution is hypo-osmotic, iso-osmotic, or hyperosmotic. A quantitative measure of osmotic pressure facilitates the dilution required to render a solution iso-osmotic relative to whole blood.

OSMOTIC PRESSURE

The osmotic pressure of a solution depends on the number of particles in solution, and is therefore referred to as a colligative property. A particle can be a molecule or an ion or an aggregated species (e.g., a dimer) that can exist discretely in solution. A solution exhibits ideal behavior when no interaction occurs between solutes and solvent, except where solvent molecules are bound to solutes by hydrogen bonding or coordinate covalency. For such a solution containing a nondissociating solute, the osmotic pressure (π) is directly proportional to its molality (number of moles of solute per kilogram of the solvent):

$$\pi = \left( \frac{\rho RT}{1000} \right) m,$$

where $\rho$ is the density of the solvent at the temperature $T$ (in the absolute scale); $R$ is the universal gas constant; and $m$ is the molality of the solution. For a real solution containing more than one solute, the osmotic pressure is given by the formula:

$$\pi = \left( \frac{\rho RT}{1000} \right) \sum \nu_i m_i \Phi_{m,i},$$

where $\nu_i$ is the number of particles formed by the dissociation of one molecule of the $i^{th}$ solute; $\nu_i = 1$ for nonionic (nondissociating) solutes; $m_i$ is the molality of the $i^{th}$ solute; and $\Phi_{m,i}$ is the molal osmotic coefficient of the $i^{th}$ solute. The molal osmotic coefficient takes into account the deviation of a solution from ideal behavior. Its value depends upon the concentration of the solute(s) in solution, its chemical properties, and ionic characteristics. The value of the molal osmotic coefficient of a solute can be determined experimentally by measuring the freezing point depression at different molal concentrations. At concentrations of pharmaceutical interest, the value of the molal osmotic coefficient is less than one. The molal osmotic coefficient decreases with the increase in concentration of the solute (Table 1).

OSMOLALITY

The osmolality of a solution $\xi_m$ is given by

$$\xi_m = \sum \nu_i m_i \Phi_{m,i}.$$

The osmolality of a real solution corresponds to the molality of an ideal solution containing nondissociating solutes and is expressed in osmoles or milliosmoles per kilogram of solvent (Osmol per kg or mOsmol per kg, respectively), a unit that is similar to the molality of the solution. Thus, osmolality is a measure of the osmotic pressure exerted by a real solution across a semipermeable membrane. Like osmotic pressure, other colligative properties of a solution, such as vapor pressure lowering, boiling point elevation, and freezing point depression, are also directly related to the osmolality of the solution. Indeed, the osmolality of a solution is typically determined most accurately and conveniently by measuring freezing point depression ($\Delta T_f$):

$$\Delta T_f = k \xi_m,$$

where $k$ is the molal cryoscopic constant, which is a property of the solvent. For water, the value of $k$ is 1.860° per Osmol. That is, 1 Osmol of a solute added to 1 kg of water lowers the freezing point by 1.860°.
OSMOLARITY

Osmolarity of a solution is a theoretical quantity expressed in osmoles per L (Osmol per L) of a solution and is widely used in clinical practice because it expresses osmoles as a function of volume. Osmolarity cannot be measured but is calculated theoretically from the experimentally measured value of osmolality.

Sometimes, osmolarity (ξ_c) is calculated theoretically from the molar concentrations:

\[ \xi_c = \sum v_i c_i \]

where \( v_i \) is as defined above, and \( c_i \) is the molar concentration of the \( i \)-th solute in solution. For example, the osmolarity of a solution prepared by dissolving 1 g of vancomycin in 100 mL of 0.9% sodium chloride solution can be calculated as follows:

\[ [3 \times 10 g/L/1449.25(\text{mol. wt. of vancomycin}) + 2 \times 9 g/L/58.44(\text{mol. wt. of sodium chloride})] \times 1000 = 329 \text{ mOsmol/L} \]

The results suggest that the solution is slightly hyperosmotic because the osmolality of blood ranges between 285 and 310 mOsmol per kg. However, the solution is found to be hypo-osmotic and has an experimentally determined osmolality of 255 mOsmol per kg. The example illustrates that osmolarity values calculated theoretically from the concentration of a solution should be interpreted cautiously and may not represent the osmotic properties of infusion solutions.

The discrepancy between theoretical (osmolarity) and experimental (osmolality) results is, in part, due to the fact that osmotic pressure is related to osmolality and not osmolarity. More significantly, the discrepancy between experimental results and the theoretical calculation is due to the fact that the osmotic pressure of a real solution is less than that of an ideal solution because of interactions between solute molecules or between solute and solvent molecules in a solution. Such interactions reduce the pressure exerted by solute molecules on a semipermeable membrane, reducing experimental values of osmolality compared to theoretical values. This difference is related to the molal osmotic coefficient (Φ_m,NaCl). The example also illustrates the importance of determining the osmolality of a solution experimentally, rather than calculating the value theoretically.

MEASUREMENT OF OSMOLALITY

The osmolality of a solution is commonly determined by the measurement of the freezing point depression of the solution.

Apparatus

The apparatus, an osmometer for freezing point depression measurement, consists of the following: a means of cooling the container used for the measurement; a resistor sensitive to temperature (thermistor), with an appropriate current- or potential-difference measurement device that may be graduated in temperature change or in osmolality; and a means of mixing the sample. Osmometers that measure the vapor pressures of solutions are less frequently employed. They require a smaller volume of specimen (generally about 5 µL), but the accuracy and precision of the resulting osmolality determination are comparable to those obtained by the use of osmometers that depend upon the observed freezing points of solutions.

Standard Solutions

Prepare Standard Solutions as specified in Table 1, as necessary.²

<table>
<thead>
<tr>
<th>Standard Solutions (Weight in g of sodium chloride per kg of water)</th>
<th>Osmolality (mOsmol/kg) (( \omega ))</th>
<th>Molal Osmotic Coefficient (Φ_m,NaCl)</th>
<th>Freezing Point Depression (°) ( \Delta T_f )</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.087</td>
<td>100</td>
<td>0.9463</td>
<td>0.186</td>
</tr>
<tr>
<td>6.260</td>
<td>200</td>
<td>0.9337</td>
<td>0.372</td>
</tr>
<tr>
<td>9.463</td>
<td>300</td>
<td>0.9264</td>
<td>0.558</td>
</tr>
<tr>
<td>12.684</td>
<td>400</td>
<td>0.9215</td>
<td>0.744</td>
</tr>
<tr>
<td>15.916</td>
<td>500</td>
<td>0.9170</td>
<td>0.930</td>
</tr>
<tr>
<td>19.147</td>
<td>600</td>
<td>0.9157</td>
<td>1.116</td>
</tr>
<tr>
<td>22.380</td>
<td>700</td>
<td>0.9140</td>
<td>1.302</td>
</tr>
</tbody>
</table>


²Commercially available solutions for osmometer calibration, with osmolalities equal to or different from those listed in Table 1 and standardized by methods traceable to NIST, may be used.
Test Solution

For a solid for injection, constitute with the appropriate diluent as specified in the instructions on the labeling. For solutions, use the sample as is. 

[NOTE—A solution can be diluted to bring it within the range of measurement of the osmometer, if necessary, but the results must be expressed as that of the diluted solution and must NOT be multiplied by a dilution factor to calculate the osmolality of the original solution, unless otherwise indicated in the monograph. The molal osmotic coefficient is a function of concentration. Therefore, it changes with dilution.]

Procedure

First, calibrate the instrument by the manufacturer’s instructions. Confirm the instrument calibration with at least one solution from Table 1 such that the osmolality of the Standard Solution lies within 50 mOsm/kg of the expected value of the Test Solution or the center of the expected range of osmolality of the Test Solution. The instrument reading should be within ±4 mOsm/kg from the Standard Solution. Introduce an appropriate volume of each Standard Solution into the measurement cell as in the manufacturer’s instructions, and start the cooling system. Usually, the mixing device is programmed to operate at a temperature below the lowest temperature expected from the freezing point depression. The apparatus indicates when the equilibrium is attained. If necessary, calibrate the osmometer, using an appropriate adjustment device such that the reading corresponds to either the osmolality or freezing point depression value of the Standard Solution shown in Table 1.

[NOTE—If the instrument reading indicates the freezing point depression, the osmolality can be derived by using the appropriate formula under Osmolality.]

Repeat the procedure with each Test Solution. Read the osmolality of the Test Solution directly, or calculate it from the measured freezing point depression.

Assuming that the value of the osmotic coefficient is essentially the same whether the concentration is expressed in molality or molarity, the experimentally determined osmolality of a solution can be converted to osmolality in the same manner in which the concentration of a solution is converted from molality to molarity. Unless a solution is very concentrated, the osmolarity can also be calculated from experimentally determined osmolality from the measurement of density of the solution. The partial specific volume of a solute is the change in volume of a solution when an additional 1 g of solute is dissolved in the solution. This volume can be calculated from the above equation correlating osmolality with osmolality that,

\[
\xi_\text{m} = 1000\xi_\text{n}/(1000/\rho + \Sigma w_i \nu_i)
\]

where \( w_i \) is the weight in g; and \( \nu_i \) is the partial specific volume, in mL per g, of the \( i \)th solute. The partial specific volume of a solute is the change in volume of a solution when an additional 1 g of solute is dissolved in the solution. This volume can be determined by the measurement of densities of the solution before and after the addition of the solute. The partial specific volumes of salts are generally very small, around 0.1 mL per g. However, those of other solutes are generally higher. For example, the partial specific volumes of amino acids are in the range of 0.6–0.9 mL per g. It can be shown from the above equation correlating osmolality with osmolality that,

\[
\xi_\text{c} = \xi_\text{m} (\rho - c)
\]

where \( \rho \) is the density of the solution, and \( c \) is the total solute concentration, both expressed in g per mL. Thus, alternatively, the osmolarity can also be calculated from experimentally determined osmolality from the measurement of density of the solution by a suitable method and the total weight of the solute, after correction for water content, dissolved per mL of the solution.

〈788〉 PARTICULATE MATTER IN INJECTIONS

This general chapter is harmonized with the corresponding texts of the European Pharmacopoeia and/or the Japanese Pharmacopoeia. These pharmacopeias have undertaken not to make any unilateral change to this harmonized chapter. Portions of the present general chapter text that are national USP text, and therefore not part of the harmonized text, are marked with symbols (*, †) to specify this fact.

Particulate matter in injections and parenteral infusions consists of extraneous mobile undissolved particles, other than gas bubbles, unintentionally present in the solutions.

*As stated in Injections and Implanted Drug Products (1), solutions for injection administered by the intramuscular or subcutaneous route must meet the requirements of Particulate Matter in Injections (788). This requirement has been indefinitely postponed for products for veterinary use. Parenterals packaged and labeled exclusively for use as irrigating solutions are exempt from the requirements of Particulate Matter in Injections (788). Radiopharmaceutical preparations are exempt from the requirements of Particulate Matter in Injections (788). Parenteral products for which the labeling specifies use of a final filter prior to administration are exempt from the requirements of Particulate Matter in Injections (788), provided that scientific data are available to justify this exemption.

For the determination of particulate matter, two procedures, Method 1 (Light Obscuration Particle Count Test) and Method 2 (Microscopic Particle Count Test), are specified hereinafter. When examining injections and parenteral infusions for subvisible particles, Method 1 is preferably applied. However, it may be necessary to test some preparations by the Light Obscuration Particle Count Test followed by the Microscopic Particle Count Test to reach a conclusion on conformance to the requirements.

Not all parenteral preparations can be examined for subvisible particles by one or both of these methods. When Method 1 is not applicable, e.g., in the case of preparations having reduced clarity or increased viscosity, the test should be carried out according to Method 2. Emulsions, colloids, and liposomal preparations are examples. Similarly, products that produce air or...
gas bubbles when drawn into the sensor may also require microscopic particle count testing. If the viscosity of the preparation to be tested is sufficiently high so as to preclude its examination by either test method, a quantitative dilution with an appropriate diluent may be made to decrease viscosity, as necessary, to allow the analysis to be performed.

The results obtained in examining a discrete unit or group of units for particulate matter cannot be extrapolated with certainty to other units that remain untested. Thus, statistically sound sampling plans must be developed if valid inferences are to be drawn from observed data to characterize the level of particulate matter in a large group of units.

*For the purpose of this chapter, small-volume parenteral is synonymous with small-volume injection, and large-volume parenteral is synonymous with large-volume injection.*

**METHOD 1 LIGHT OBSCURATION PARTICLE COUNT TEST**

Use a suitable apparatus based on the principle of light blockage that allows for an automatic determination of the size of particles and the number of particles according to size. The definition for particle-free water is provided in Reagents, Indicators, and Solutions—Reagent Specifications.

The apparatus is calibrated using dispersions of spherical particles of known sizes between 10 µm and 25 µm. These standard particles are dispersed in particle-free water. Care must be taken to avoid aggregation of particles during dispersion. *System suitability can be verified by using the USP Particle Count RS.*

**General Precautions**

The test is carried out under conditions limiting particulate matter, preferably in a laminar flow cabinet. Very carefully wash the glassware and filtration equipment used, except for the membrane filters, with a warm detergent solution, and rinse with abundant amounts of water to remove all traces of detergent. Immediately before use, rinse the equipment from top to bottom, outside and then inside, with particle-free water.

Take care not to introduce air bubbles into the preparation to be examined, especially when fractions of the preparation are being transferred to the container in which the determination is to be carried out.

In order to check that the environment is suitable for the test, that the glassware is properly cleaned, and that the water to be used is particle-free, the following test is carried out. Determine the particulate matter in five samples of particle-free water, each of 5 mL, according to the method described below. If the number of particles of 10 µm or greater size exceeds 25 for the combined 25 mL, the precautions taken for the test are not sufficient. The preparatory steps must be repeated until the environment, glassware, and water are suitable for the test.

**Method**

Mix the contents of the sample by slowly inverting the container 20 times successively. If necessary, cautiously remove the sealing closure. Clean the outer surfaces of the container opening using a jet of particle-free water, and remove the closure, avoiding any contamination of the contents. Eliminate gas bubbles by appropriate measures such as allowing to stand for 2 min or sonicating.

For large-volume parenterals, single units are tested. For small-volume parenterals less than 25 mL in volume, the contents of 10 or more units are combined in a cleaned container to obtain a volume of NLT 25 mL; the test solution may be prepared by mixing the contents of a suitable number of vials and diluting to 25 mL with particle-free water or with an appropriate particle-free solvent when particle-free water is not suitable. Small-volume parenterals having a volume of 25 mL or more may be tested individually.

Powders for parenteral use are reconstituted with particle-free water or with an appropriate particle-free solvent when particle-free water is not suitable.

For pharmacy bulk packages for parenteral use labeled “Not for Direct Infusion”, proceed as directed for small-volume parenterals when the volume is 25 mL or more. Calculate the test result on a portion that is equivalent to the maximum dose given in the labeling. For example, if the total bulk package volume is 100 mL and the maximum dose volume is 10 mL, then the average particle count per mL would be multiplied by 10 to obtain the test result based on the 10-mL maximum dose. [Note—For the calculation of test results, consider this maximum dose portion to be equivalent to the contents of one full container.]

Products packaged with dual compartments meant to hold a drug product and a solvent should be prepared and tested as directed for large-volume parenterals or small-volume parenterals, depending on container volume. Mix each unit as directed in the labeling, activating and agitating to ensure thorough mixing of the separate components and drug dissolution.

The number of test specimens must be adequate to provide a statistically sound assessment. For large-volume parenterals or for small-volume parenterals having a volume of 25 mL or more, fewer than 10 units may be tested, using an appropriate sampling plan.

Remove four portions, NLT 5 mL each, and count the number of particles equal to or greater than 10 µm and 25 µm. Disregard the result obtained for the first portion, and calculate the mean number of particles for the preparation to be examined.

**Evaluation**

For preparations supplied in containers with a nominal volume of more than 100 mL, apply the criteria of Test 1.A. For preparations supplied in containers with a nominal volume of less than 100 mL, apply the criteria of Test 1.B. [Note—Test 1.A is used in the Japanese Pharmacopoeia.]
If the average number of particles exceeds the limits, test the preparation by the Microscopic Particle Count Test.

**TEST 1.A**

**Solutions for parenteral infusion or solutions for injection supplied in containers with a nominal content of more than 100 mL:** The preparation complies with the test if the average number of particles present in the units tested does not exceed 25 per mL equal to or greater than 10 µm and does not exceed 3 per mL equal to or greater than 25 µm.

**TEST 1.B**

**Solutions for parenteral infusion or solutions for injection supplied in containers with a nominal content of less than 100 mL:** The preparation complies with the test if the average number of particles present in the units tested does not exceed 6000 per container equal to or greater than 10 µm and does not exceed 600 per container equal to or greater than 25 µm.

**METHOD 2 MICROSCOPIC PARTICLE COUNT TEST**

Use a suitable binocular microscope, a filter assembly for retaining particulate matter, and a membrane filter for examination. The microscope is adjusted to 100 ± 10 magnifications and is equipped with an ocular micrometer calibrated with an objective micrometer, a mechanical stage capable of holding and traversing the entire filtration area of the membrane filter, and two suitable illuminators to provide episcopic illumination in addition to oblique illumination.

The ocular micrometer is a circular diameter graticule (see Figure 1) and consists of a large circle divided by crosshairs into quadrants, transparent and black reference circles 10 µm and 25 µm in diameter at 100 magnifications, and a linear scale graduated in 10-µm increments. It is calibrated using a stage micrometer that is certified by either a domestic or international standard institution. A relative error of the linear scale of the graticule within ±2% is acceptable. The large circle is designated the graticule field of view (GFOV).

![Figure 1. Circular diameter graticule. The large circle divided by crosshairs into quadrants is designated the graticule field of view (GFOV). Transparent and black circles having 10-µm and 25-µm diameters at 100× are provided as comparison scales for particle sizing.](image)

Two illuminators are required. One is an episcopic brightfield illuminator internal to the microscope, the other is an external, focusable auxiliary illuminator that can be adjusted to give reflected oblique illumination at an angle of 10°–20°.

The filter assembly for retaining particulate matter consists of a filter holder made of glass or other suitable material, and is equipped with a vacuum source and a suitable membrane filter.

The membrane filter is of suitable size, black or dark gray in color, nongridded or gridded, and 1.0 µm or finer in nominal pore size.

**General Precautions**

The test is carried out under conditions limiting particulate matter, preferably in a laminar flow cabinet.

Very carefully wash the glassware and filter assembly used, except for the membrane filter, with a warm detergent solution, and rinse with abundant amounts of water to remove all traces of detergent. Immediately before use, rinse both sides of the membrane filter and the equipment from top to bottom, outside and then inside, with particle-free water.

In order to check that the environment is suitable for the test, that the glassware and the membrane filter are properly cleaned, and that the water to be used is particle-free, the following test is carried out. Determine the particulate matter of a 50-mL volume of particle-free water according to the method described below. If more than 20 particles 10 µm or larger in size or if more than five particles 25 µm or larger in size are present within the filtration area, the precautions taken for the test are...
not sufficient. The preparatory steps must be repeated until the environment, glassware, membrane filter, and water are suitable for the test.

**Method**

Mix the contents of the samples by slowly inverting the container 20 times successively. If necessary, cautiously remove the sealing closure. Clean the outer surfaces of the container opening using a jet of particle-free water, and remove the closure, avoiding any contamination of the contents.

For large-volume parenterals, single units are tested. For small-volume parenterals less than 25 mL in volume, the contents of 10 or more units are combined in a clean container; the test solution may be prepared by mixing the contents of a suitable number of vials and diluting to 25 mL with particle-free water or with an appropriate particle-free solvent when particle-free water is not suitable. Small-volume parenterals having a volume of 25 mL or more may be tested individually.

Powders for parenteral use are constituted with particle-free water or with an appropriate particle-free solvent when particle-free water is not suitable.

The number of test specimens must be adequate to provide a statistically sound assessment. For large-volume parenterals or for small-volume parenterals having a volume of 25 mL or more, fewer than 10 units may be tested, using an appropriate sampling plan.

Wet the inside of the filter holder fitted with the membrane filter with several mL of particle-free water. Transfer to the filtration funnel the total volume of a solution pool or of a single unit, and apply a vacuum. If needed, add stepwise a portion of the solution until the entire volume is filtered. After the last addition of solution, begin rinsing the inner walls of the filter holder by using a jet of particle-free water. Maintain the vacuum until the surface of the membrane filter is free from liquid. Place the membrane filter in a Petri dish, and allow the membrane filter to air-dry with the cover slightly ajar. After the membrane filter has been dried, place the Petri dish on the stage of the microscope, scan the entire membrane filter under the reflected light from the illuminating device, and count the number of particles that are equal to or greater than 10 μm and the number of particles that are equal to or greater than 25 μm. Alternatively, partial membrane filter count and determination of the total filter count by calculation is allowed. Calculate the mean number of particles for the preparation to be examined.

The particle sizing process with the use of the circular diameter graticule is carried out by estimating the equivalent diameter of the particle in comparison with the 10 μm and 25 μm reference circles on the graticule. Thereby the particles are not moved from their initial locations within the graticule field of view and are not superimposed on the reference circles for comparison. The inner diameter of the transparent graticule reference circles is used to size white and transparent particles, while dark particles are sized by using the outer diameter of the black opaque graticule reference circles.

In performing the Microscopic Particle Count Test, do not attempt to size or enumerate amorphous, semiliquid, or otherwise morphologically indistinct materials that have the appearance of a stain or discoloration on the membrane filter. These materials show little or no surface relief and present a gelatinous or film-like appearance. In such cases, the interpretation of enumeration may be aided by testing a sample of the solution by the Light Obscuration Particle Count Test.

**Evaluation**

For preparations supplied in containers with a nominal volume of more than 100 mL, apply the criteria of Test 2.A. For preparations supplied in containers with a nominal volume of less than 100 mL, apply the criteria of Test 2.B.

**OTE—Test 2.A is used in the Japanese Pharmacopoeia.**

**TEST 2.A**

**Solutions for parenteral infusion or solutions for injection supplied in containers with a nominal content of more than 100 mL:** The preparation complies with the test if the average number of particles present in the units tested does not exceed 12 per mL equal to or greater than 10 μm and does not exceed 2 per mL equal to or greater than 25 μm.

**TEST 2.B**

**Solutions for parenteral infusion or solutions for injection supplied in containers with a nominal content of less than 100 mL:** The preparation complies with the test if the average number of particles present in the units tested does not exceed 3000 per container equal to or greater than 10 μm and does not exceed 300 per container equal to or greater than 25 μm.

**〈791〉 pH**

**INTRODUCTION**

For compendial purposes, pH is defined as the value given by a suitable, properly calibrated, potentiometric sensor and measuring system. [Note—The measuring system has traditionally been referred to as the “pH meter.” While the pH meter is still in common use, the measuring system can also be embedded inside the pH sensor, and the pH signal can be transmitted digitally to an external device such as a computer, Programmable Logic Controller (PLC), Distributed Control System (DCS), data acquisition system, terminal, or other microprocessor-controlled device.] By definition, pH is equal to \(-\log_{10}[a_{\text{H}^+}]\) where \(a_{\text{H}^+}\) is the activity of the hydrogen (H+) or hydronium ion (H3O+), and the hydrogen ion activity very closely approximates the hydrogen ion concentration.

Published on March 26, 2020
The practical pH scale is defined:

\[ \text{pH} = \text{pH}_S + \left( \frac{(E - E_S)}{k} \right) \]

- \( E \) = measured potential where the galvanic cell contains the solution under test (pH)
- \( E_S \) = measured potential where the galvanic cell contains the appropriate buffer solution for calibration (pH)
- \( k \) = change in potential/unit change in pH and is derived from the Nernst equation (as follows)

\[ k = \log_{10}(10) \times \left( \frac{RT}{nF} \right) \]

\( R = 8.314 \text{ J/mole/}°\text{K} \)
\( T = \text{temperature (°K)} \)
\( n = \text{moles/half-reaction} \)
\( F = \text{Faraday constant, 96485 C/mole} \)

The resulting equation is \[0.05916 + 0.0001984(T - 25°)\] volts at temperature \( T \). Values of \( k \) from 15°–35° are provided in Table 1.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>( k ) (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15.00</td>
<td>0.05718</td>
</tr>
<tr>
<td>20.00</td>
<td>0.05817</td>
</tr>
<tr>
<td>25.00</td>
<td>0.05916</td>
</tr>
<tr>
<td>30.00</td>
<td>0.06016</td>
</tr>
<tr>
<td>35.00</td>
<td>0.06115</td>
</tr>
</tbody>
</table>

Values of \( k \) at other temperatures can be determined from the equation above. For practical purposes, values of \( k \) are determined from pH sensor calibration.

**pH MEASUREMENT SYSTEM**

The measurement system consists of 1) a measuring electrode sensitive to hydrogen-ion activity, typically a glass electrode, though other electrode types are possible; 2) a suitable reference electrode, for example, a silver-silver chloride electrode; and 3) a voltage measurement system with an input resistance capable of measuring at a high input impedance of the pH sensor. The measuring and reference electrode may be separated or combined. The voltage measurement system may be separated from the pH sensor or integrated into the sensor. For most applications, a temperature measurement will be necessary for compensation of the Nernst temperature influence described above. A temperature device may be embedded into the pH sensor, or an external temperature device may be used.

**INSTRUMENT REQUIREMENTS**

The measurement system shall be capable of performing a two-point (or more) pH calibration (see below). The accuracy of the pH measurement system is described in the Calibration section. The resolution of the pH measurement system shall be at least 0.01 pH. The instrument shall be capable of temperature-compensating the pH sensor measurement to convert the millivolt signal to pH units at any temperature, either automatically using a temperature device built into the sensor system or by manual entry of the sample temperature into the measurement system. The accuracy of the temperature measurement system shall be ±1°. The resolution of the temperature measurement system shall be at least 0.1°. Lab-based pH measurements are typically performed at 25 ± 2° unless otherwise specified in the individual monograph or herein. However, temperatures outside this range are acceptable if samples are more conveniently prepared at alternative temperatures. Examples of non-lab-based measurements include test samples inside process pipes, vessels, tanks, and other non-standard processing conditions. [Note—The definitions of pH, the pH scale, and the values assigned to the buffer solutions for calibration are for the purpose of establishing a practical, operational system so that results may be compared between laboratories. The measured pH values do not necessarily correspond exactly to those obtained by the definition, pH = −log_{10} [a_{H^+}], rather the values obtained are closely related to the activity of the hydrogen-ion in aqueous solutions.] [Note—Where a pH measurement system is calibrated by use of an aqueous buffer and then used to measure the pH of nonaqueous systems, the ionization constant of the acid or base, the dielectric constant of the medium, the liquid-junction potential (which may give rise to errors of approximately 1 pH unit), and the hydrogen-ion response of the glass electrode are all changed. For these reasons, the values so obtained with solutions that are only partially aqueous in character can be regarded only as apparent pH values.]
BUFFERS FOR CALIBRATION OF THE pH MEASUREMENT SYSTEM

Buffer solutions for calibration are prepared as directed in Table 2. Buffer salts of requisite purity can be obtained from the National Institute of Standards and Technology (NIST), other national authorities, or other suppliers. Buffer solutions should be stored in appropriate containers that ensure stability of the pH through the expiry date, and fitted with a tight closure. For buffer solutions greater than 11, the storage should be in containers that are resistant to or reduce carbon dioxide intrusion, which would lower the pH of the buffer. For buffer solutions lower than 11, they should typically be prepared at intervals not to exceed 3 months. For buffer solutions greater than 11, they should typically be prepared and used fresh unless carbon dioxide ingress is restricted. All buffer solutions should be prepared using purified water. Table 2 indicates the pH of the buffer solutions as a function of temperature. The instructions presented here are intended for the preparation of solutions having the designated molar (m) concentrations. However, in order to facilitate their preparation, the instructions are given in molarity. The difference in concentration between molality and molarity preparations for these buffer solutions is less than 1%, and the pH difference is negligible. Calibration using buffer solutions shall be done in the temperature range of the buffers listed in Table 2. [NOTE—The Nernst temperature compensation corrects only for the electrode millivolt output change with temperature, not the actual pH change of the buffer solution with temperature, which is unique for each buffer.] Features such as automatic buffer recognition or buffer pH–temperature correction are available for convenience in accommodating the temperature influence on buffer solutions. The pH–temperature response can be determined from the values in Table 2.

Table 2. pH Values of Buffer Solutions for Calibration

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Potassium Tetraoxalate, 0.05 m</th>
<th>Potassium Hydrogen Tartrate, Saturated at 25°</th>
<th>Potassium Dihydrogen Citrate, 0.05 M</th>
<th>Equimolal Phosphate, 0.05 m</th>
<th>Potassium Dihydrogen Phosphate, 0.0087 M and Disodium Hydrogen Phosphate, 0.0303 M</th>
<th>Sodium Tetaborate, 0.01 M</th>
<th>Sodium Carbonate, 0.025 M and Sodium Bicarbonate, 0.025 M</th>
<th>Calcium Hydroxide, Saturated at 25°</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>1.67</td>
<td>—</td>
<td>4.00</td>
<td>6.92</td>
<td>9.33</td>
<td>—</td>
<td>13.00</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>1.67</td>
<td>3.80</td>
<td>4.00</td>
<td>6.90</td>
<td>7.45</td>
<td>9.28</td>
<td>10.12</td>
<td>12.81</td>
</tr>
<tr>
<td>20</td>
<td>1.68</td>
<td>3.79</td>
<td>4.00</td>
<td>6.88</td>
<td>7.43</td>
<td>9.23</td>
<td>10.06</td>
<td>12.63</td>
</tr>
<tr>
<td>25</td>
<td>1.68</td>
<td>3.56</td>
<td>3.78</td>
<td>4.01</td>
<td>6.86</td>
<td>7.41</td>
<td>9.18</td>
<td>10.01</td>
</tr>
<tr>
<td>35</td>
<td>1.69</td>
<td>3.55</td>
<td>3.76</td>
<td>4.02</td>
<td>6.84</td>
<td>7.39</td>
<td>9.10</td>
<td>9.93</td>
</tr>
<tr>
<td>40</td>
<td>1.69</td>
<td>—</td>
<td>4.04</td>
<td>6.84</td>
<td>—</td>
<td>9.07</td>
<td>—</td>
<td>11.98</td>
</tr>
<tr>
<td>45</td>
<td>1.70</td>
<td>—</td>
<td>4.05</td>
<td>6.83</td>
<td>—</td>
<td>9.04</td>
<td>—</td>
<td>11.84</td>
</tr>
<tr>
<td>50</td>
<td>1.71</td>
<td>—</td>
<td>4.06</td>
<td>6.83</td>
<td>—</td>
<td>9.01</td>
<td>—</td>
<td>11.71</td>
</tr>
<tr>
<td>55</td>
<td>1.72</td>
<td>—</td>
<td>4.08</td>
<td>6.83</td>
<td>—</td>
<td>8.99</td>
<td>—</td>
<td>11.57</td>
</tr>
<tr>
<td>60</td>
<td>1.72</td>
<td>—</td>
<td>4.09</td>
<td>6.84</td>
<td>—</td>
<td>8.96</td>
<td>—</td>
<td>11.45</td>
</tr>
</tbody>
</table>

ΔpH/°C = 0.0010 – 0.0014 – 0.0022 0.0018 – 0.0016 – 0.0028 – 0.0074 – 0.0096 – 0.0310

Preparation of alternative volumes at the same concentrations to those indicated below is acceptable.

- **Potassium tetraoxalate, 0.05 m**: Dissolve 12.61 g of KH₂(C₆O₆)₂ · 2H₂O and dilute with water to make 1000.0 mL.
- **Potassium hydrogen tartrate, saturated at 25°**: Add C₄H₄KO₄ to water until saturation is exceeded at 25°. Then filter or decant.
- **Potassium biphthalate, 0.05 m**: Dissolve 10.12 g of KH₂(C₆H₇O₇)₂ previously dried at 110° for 1 h and dilute with water to make 1000.0 mL.
- **Equimolal phosphate, 0.05 m**: Dissolve 3.53 g of disodium hydrogen phosphate (Na₂HPO₄) and 3.39 g of potassium dihydrogen phosphate (KH₂PO₄), each previously dried at 120° for 2 h, and dilute with water to make 1000.0 mL.
- **Potassium dihydrogen phosphate, 0.0087 M and disodium hydrogen phosphate, 0.0303 M**: Dissolve 1.18 g of KH₂PO₄ and 4.30 g of Na₂HPO₄ both dried for 2 h at 120 ± 2°, and dilute with water to make 1000.0 mL.
- **Sodium tetaborate, 0.01 M**: Dissolve 3.80 g of Na₂B₄O₇ · 10H₂O, and dilute with water to make 1000.0 mL. Protect from absorption of carbon dioxide.
- **Sodium carbonate, 0.025 M and sodium bicarbonate, 0.025 M**: Dissolve 2.64 g of sodium carbonate (Na₂CO₃) and 2.09 g of sodium bicarbonate (NaHCO₃), and dilute with water to make 1000.0 mL.

1 Commercially available buffer solutions for pH measurement system, calibrated by methods traceable to NIST or other national authorities, labeled with a pH value accurate to 0.02 pH units may be used. Solutions prepared from ACS reagent-grade materials or other suitable materials, may be used provided the pH of the resultant solution is the same as that of the solution prepared from the NIST (or other national authorities)-certified material. Buffer solutions that are greater than 12 should be used immediately or should be prepared using freshly boiled water, and they should be stored under conditions to minimize carbon dioxide absorption and ingress.
CALIBRATION

Because of variations in the nature and operation of the available pH measurement systems, it is not practical to provide universal directions for the calibration of the measurement system. However, the general principles to be followed are set forth in the following paragraphs. Examine the electrodes, especially the reference electrode and electrolyte level, if a liquid electrolyte is used. If necessary, replenish electrolyte supply, and observe other precautions indicated by the instrument and electrode manufacturers.

The calibration or verification of the pH measurement system should be periodically executed. The historical performance of the measurement system, the criticality of the pH measurement, the maintenance of the pH sensor, and the frequency of measurement operation are used to determine the frequency of the calibration/verification.

The procedure below allows for several calibration methodologies (two-point calibration, multiple-point calibration, and multiple-segment calibration).

If the pH of the buffer is sensitive to ambient carbon dioxide, then use Purified Water that has been recently boiled, and subsequently stored in a container designed to minimize ingress of carbon dioxide.

1. To calibrate the pH measurement system, select three buffer solutions for calibration, preferably from those given in Table 2, such that the expected pH of the material under test falls within their range. Two of the buffers are used for the calibration process, and the third buffer is used for verification. The value of the verification buffer shall be between two of the calibration buffers. If the operational range of the pH sensor is beyond the pH range of the buffer solutions in Table 2, then either 1) select two nearby pH buffers from Table 2 or 2) select one from Table 2 and another documented prepared buffer that is outside the range.

2. Rinse the pH sensor several times with water, then with the first buffer solution.

3. Immerse the pH sensor in the first buffer solution at a temperature within the range of Table 2.

4. If automatic temperature measurement and compensation are not included in the measuring system, manually enter the temperature of the buffer and pH value of the buffer solution at that temperature into the instrument. For temperatures not listed in Table 2, use linear interpolation to determine the pH value as a function of temperature.

5. Initiate the two-point calibration sequence with the first buffer according to the manufacturer’s instructions.

6. Remove the pH sensor from the first buffer and rinse the electrode(s) with water, and then with the second buffer solution.

7. Immerse the pH sensor in the second buffer at a temperature within the range of Table 2.

8. If automatic temperature measurement and compensation are not included in the measuring system, manually enter the temperature of the buffer and pH value of the buffer solution at that temperature into the instrument.

9. Continue the two-point calibration sequence with the second buffer according to the manufacturer's instructions.

10. After completion of the two-point calibration process, verify that the pH slope and offset are within acceptable parameters. Typical acceptable parameters are a slope of 90%–105% and an offset of 0 ± 30 mV (0.5 pH units at 25°C). Depending on the pH instrumentation, the pH slope and offset may be determined in software or by manual methods. If using manual methods, follow supplier instructions to calculate the pH sensor slope/offset. If these parameters are not within acceptable parameters, the sensor should be properly cleaned, replenished, serviced, or replaced, and the two-point calibration process shall be repeated.

11. Remove the pH sensor from the second buffer, and rinse thoroughly with water, and then the verification buffer.

12. Immerse the pH sensor in the verification buffer at a temperature within the range of Table 2.

13. If automatic temperature measurement and compensation are not included in the measuring system, manually enter the temperature of the buffer and pH value of the buffer solution at that temperature into the instrument.

14. The pH reading shall be within ±0.05 pH of the value in Table 2 at the buffer solution temperature.

NOTE—If a multiple-point calibration process (three or more calibration buffers) plus at least one verification buffer are used, then repeat steps 9–14, assuring that the pH sensor slope and offset criteria (see step 10) and the calibration accuracy (see step 14) of this range are met. The value of the verification buffers shall be between the highest and lowest calibration buffers of the range.

NOTE—If a multiple-segment calibration process (three or more calibration buffers with at least two slopes and offsets) plus at least one verification buffer for each segment are used, then repeat steps 9–14 for each segment, assuring that the pH sensor slope and offset criteria (see step 10) and the calibration accuracy (see step 14) of each segment are met. The value of each verification buffer shall be between the highest and lowest calibration buffers for each segment.

OPERATION

All test samples should be prepared using Purified Water, unless otherwise specified in the monograph. All test measurements should use manual or automated Nernst temperature compensation.

1. Prepare the test material according to requirements in the monograph or according to specific procedures. If the pH of the test sample is sensitive to ambient carbon dioxide, then use Purified Water that has been recently boiled, and subsequently stored in a container designed to minimize ingress of carbon dioxide.

2. Rinse the pH sensor with water, then with a few portions of the test material.
3. Immerse the pH sensor into the test material and record the pH value and temperature. In all pH measurements, allow sufficient time for stabilization of the temperature and pH measurement. Diagnostic functions such as glass or reference electrode resistance measurement may be available to determine equipment deficiencies. Refer to the electrode supplier for diagnostic tools to assure proper electrode function. Where approximate pH values suffice, indicators and test papers (see Reagents, Indicators, and Solutions—Indicators and Indicator Test Papers, Indicators and Indicator and Test Papers) may be suitable.

For a discussion of buffers, and for the composition of standard buffer solutions called for in compendial tests and assays, see Reagents, Indicators, and Solutions—Solutions, Buffer Solutions. This referenced section is not intended to replace the use of the pH calibration buffers in Table 2.

Change to read:

▲ PHARMACEUTICAL COMPOUNDING—NONSTERILE PREPARATIONS

The official date for this chapter is postponed until further notice. When the official date is reestablished, the period allowed for implementation will not be less than six months.

1. INTRODUCTION AND SCOPE
   1.1 Scope

2. PERSONNEL TRAINING AND EVALUATION

3. PERSONAL HYGIENE AND GARBLING
   3.1 Personnel Preparation
   3.2 Hand Hygiene
   3.3 Garb and Glove Requirements

4. BUILDINGS AND FACILITIES
   4.1 Compounding Space
   4.2 Storage Area
   4.3 Water Sources

5. CLEANING AND SANITIZING

6. EQUIPMENT AND COMPONENTS
   6.1 Equipment
   6.2 Components

7. MASTER FORMULATION AND COMPOUNDING RECORDS
   7.1 Creating Master Formulation Records
   7.2 Creating Compounding Records

8. RELEASE INSPECTIONS

9. LABELING

10. ESTABLISHING BEYOND-USE DATES
    10.1 Terminology
    10.2 Parameters to Consider in Establishing a BUD
    10.3 Establishing a BUD for a CNSP
    10.4 CNSPs Requiring Shorter BUDs
    10.5 Extending BUDs for CNSPs

11. SOPs

12. QUALITY ASSURANCE AND QUALITY CONTROL

13. CNSP PACKAGING AND TRANSPORTING
    13.1 Packaging of CNSPs
    13.2 Transporting CNSPs

14. COMPLAINT HANDLING AND ADVERSE EVENT REPORTING
    14.1 Complaint Handling
    14.2 Adverse Event Reporting

15. DOCUMENTATION

GLOSSARY

APPENDIX

1. INTRODUCTION AND SCOPE

This chapter describes the minimum standards to be followed when preparing compounded nonsterile preparations (CNSPs) for humans and animals. For purposes of this chapter, nonsterile compounding is defined as combining, admixing, diluting,
pooling, reconstituting other than as provided in the manufacturer’s labeling, or otherwise altering a drug or bulk drug substance to create a nonsterile medication.

The requirements in this chapter must be followed to minimize harm, including death, to human and animal patients that could result from 1) excessive microbial contamination, 2) variability from the intended strength of correct ingredients (e.g., ±10% of the labeled strength), 3) physical and chemical incompatibilities, 4) chemical and physical contaminants, and/or 5) use of ingredients of inappropriate quality.

Handling of nonsterile hazardous drugs (HDs) must additionally comply with Hazardous Drugs—Handling in Healthcare Settings (800).

1.1 Scope

CNSPs SUBJECT TO THE REQUIREMENTS IN THIS CHAPTER

CNSPs that must comply with this chapter include but are not limited to the following dosage forms:

- Solid oral preparations
- Liquid oral preparations
- Rectal preparations
- Vaginal preparations
- Topical preparations (i.e., creams, gels, ointments)
- Nasal and sinus preparations intended for local application (i.e., nasal sprays and nasal irrigation)
- Otic preparations

PRACTICES NOT SUBJECT TO THE REQUIREMENTS IN THIS CHAPTER

The following practices are not considered compounding and are not required to meet the requirements of this chapter:

Administration: Preparation of a single dose for a single patient when administration will begin within 4 hours of beginning the preparation is not required to meet the standards in this chapter.

Nonsterile radiopharmaceuticals: Compounding of nonsterile radiopharmaceuticals is not required to meet the standards in this chapter and is subject to the requirements in Radiopharmaceuticals—Preparation, Compounding, Dispensing, and Repackaging (825).

Reconstitution: Reconstitution of a conventionally manufactured nonsterile product in accordance with the directions contained in the manufacturer approved labeling is not required to meet the standards in this chapter.

Repackaging: Repackaging of conventionally manufactured drug products is not required to meet the standards in this chapter (see Good Repackaging Practices (1178)).

Splitting tablets: Breaking or cutting a tablet into smaller portions is not required to meet the standards in this chapter.

PERSONNEL AND SETTINGS AFFECTED

This chapter applies to all persons who prepare CNSPs and all places where CNSPs are prepared. This includes but is not limited to pharmacists, technicians, nurses, physicians, veterinarians, dentists, naturopaths, and chiropractors, in all places including but not limited to pharmacies, hospitals and other healthcare institutions, patient treatment sites, and physicians’ or veterinarians’ practice sites.

The compounding facility’s leadership and all personnel involved in preparing, storing, packaging, and transporting CNSPs are responsible for 1) ensuring that the applicable practices and quality standards in this chapter are continually and consistently applied to their operations, and 2) proactively identifying and remedying potential problems within their operations. Personnel engaged in the compounding of CNSPs must also comply with laws and regulations of the applicable regulatory jurisdiction.

The compounding facility must designate one or more individuals to be responsible and accountable for the performance and operation of the facility and personnel for the preparation of CNSPs. The responsibilities of the designated person(s) include but are not limited to:

- Overseeing a training program to ensure competency of personnel involved in compounding, handling, and preparing of CNSPs
- Selecting components
- Monitoring and observing compounding activities and taking immediate corrective action if deficient practices are observed
- Ensuring that standard operating procedures (SOPs) are fully implemented. The designated person(s) must ensure that follow-up is carried out if problems, deviations, or errors are identified
- Establishing, monitoring, and documenting procedures for the handling and storage of CNSPs and/or components of CNSPs

The designated person(s) must be identified in an SOP. If the compounding facility has only one person responsible for all of the compounding in the facility, then that person is the designated person.
2. PERSONNEL TRAINING AND EVALUATION

All personnel involved in the preparation and handling of CNSPs must be initially trained, must demonstrate competency, and must undergo refresher training every 12 months. Training and competency of personnel must be documented as described in 15. Documentation.

A designated person must oversee a training program that describes the required training, the frequency of training, and the process for evaluating the competency of personnel involved in nonsterile compounding and handling of CNSPs. This program must equip personnel with knowledge and training in the required skills necessary to perform their assigned tasks.

Before beginning to prepare CNSPs independently, all compounding personnel must complete training and be able to demonstrate proficiency in the principles and hands-on skills of nonsterile manipulations for the type of compounding they will be performing. Proficiency must be demonstrated every 12 months in at least the following core competencies:

- Hand hygiene
- Garbing
- Cleaning and sanitizing
- Handling and transporting components and CNSPs
- Measuring and mixing
- Proper use of equipment and devices selected to compound CNSPs
- Documentation of the compounding process (e.g., Master Formulation Records and Compounding Records)

Steps in the training procedure must include the following:

- Read and understand this chapter, other applicable standards, and other relevant literature
- Understand and interpret Safety Data Sheets (SDSs) and, if applicable, Certificates of Analysis (COA)
- Read and understand procedures related to their compounding duties

A designated person must oversee the training of personnel. Training and observation may be performed by the designated person(s) or an assigned trainer. Personnel must be observed and guided throughout the training process. The personnel will then be expected to repeat the procedures independently, but under the direct supervision of the designated person(s) and/or trainer. Personnel will be permitted to perform the procedure without direct supervision only after independently demonstrating understanding and competency. Upon completion of the training program, the designated person(s) and/or trainer must document that the personnel has been trained and successfully completed competency assessments (see 15. Documentation).

In addition to the initial and annual competency training and evaluation described in this section, a designated person should monitor and observe compounding activities and must take immediate corrective action if deficient practices are observed. SOPs must describe procedures for the monitoring and observing of compounding activities and personnel.

If the facility has only one person in the compounding operation, that person must document that they have obtained training and demonstrated competency, and they must comply with the other requirements of this chapter.

3. PERSONAL HYGIENE AND GARBING

Individuals entering the compounding area must maintain personal hygiene. Individuals must evaluate whether they have a personal risk of potentially contaminating the compounding environment and CNSP (e.g., personnel with rashes, recent tattoos or oozing sores, conjunctivitis, or active respiratory infection). Individuals must report these conditions to the designated person(s). The designated person(s) is responsible for evaluating whether these individuals should be excluded from working in compounding areas before their conditions have resolved because of the risk of contaminating the CNSP and the environment.

3.1 Personnel Preparation

Personnel engaged in compounding must maintain hand hygiene and maintain cleanliness required for the type of compounding performed.

Before entering the compounding area, compounding personnel must remove any items that are not easily cleanable and that might interfere with garbing. At a minimum, personnel must:

- Remove personal outer garments (e.g., bandanas, coats, hats, jackets)
- Remove all hand, wrist, and other exposed jewelry, including piercings that could interfere with the effectiveness of garbing or hand hygiene (e.g., watches, rings that may tear gloves)
- Remove earbuds or headphones

The designated person(s) may permit accommodations as long as the quality of the environment and CNSP will not be affected.

3.2 Hand Hygiene

Personnel must perform hand hygiene when entering the compounding area to compound as described in Box 3-1. Alcohol hand sanitizers alone are not sufficient.
Box 3-1. Hand Hygiene Procedures

- Wash hands and forearms up to the elbows with soap and water for at least 30 seconds.
- Dry hands and forearms to the elbows completely with disposable towels or wipers.
- Allow hands and forearms to dry thoroughly before donning gloves.

To minimize the risk of cross-contaminating other CNSPs and contaminating other objects (e.g., pens and keyboards), gloves should be wiped or replaced before beginning a CNSP with different components.

All gloves must be inspected for holes, punctures, or tears and must be replaced immediately if such defects are detected.

3.3 Garb and Glove Requirements

Gloves must be worn for all compounding activities. Other garb (e.g., shoe covers, head and facial hair covers, face masks, gowns) should be worn as needed for the protection of personnel from chemical exposures and for prevention of preparation contamination and must be appropriate for the type of compounding performed. The garbing requirements and frequency of changing the garb must be determined by the facility and documented in the facility’s SOPs.

Garb must be stored in a manner that minimizes contamination (e.g., away from sinks to avoid splashing). Visibly soiled garb or garb with tears or punctures must be changed immediately.

If gowns are worn, they may be re-used if not soiled. If used, gloves, shoe covers, hair covers, facial hair covers, face masks, or head coverings may not be re-used and must be replaced with new ones. If used, non-disposable garb, such as goggles, should be cleaned and sanitized with 70% isopropyl alcohol before re-use.

4. BUILDINGS AND FACILITIES

4.1 Compounding Space

Space must be specifically designated for nonsterile compounding. The method of designation (e.g., visible perimeter) must be described in the facility’s SOP. Other activities must not be occurring in the space at the same time as compounding. The compounding space must be well-lighted and must be maintained in a clean, orderly, and sanitary condition, and in a good state of repair. Carpet is not allowed in the compounding space. Surfaces should be resistant to damage by cleaning and sanitizing agents.

The space must provide for the orderly placement of equipment and materials to prevent mix-ups among components, containers, labels, in-process materials, and finished CNSPs. The space should be designed, arranged, and used in a way that minimizes cross-contamination from non-compounding areas.

4.2 Storage Area

Compounding personnel must monitor temperatures in storage area(s) either manually at least once daily on days that the facility is open or by a continuous temperature recording device to determine whether the temperature remains within the appropriate range for the CNSPs or components. The results of the temperature readings must be documented on a temperature log or stored in the continuous temperature recording device, and must be retrievable. All temperature monitoring equipment must be calibrated or verified for accuracy as recommended by the manufacturer or every 12 months if not specified by the manufacturer.

The compounding facility must adhere to SOPs to detect and prevent temperature excursions within storage area(s). When it is known that a CNSP or component has been exposed to temperatures either below or above the storage temperature limits for the CNSP or component, personnel must determine whether the CNSP or component integrity or quality has been compromised and, if so, the CNSP or component must be discarded.

All CNSPs, components, equipment, and containers must be stored off the floor and in a manner that prevents contamination and permits inspection and cleaning of the storage area(s).

4.3 Water Sources

A source of hot and cold water and an easily accessible sink must be available for compounding. The sink must be emptied of all items unrelated to compounding and cleaned when visibly soiled before being used to clean any equipment used in nonsterile compounding. The plumbing system must be free of defects that may contribute to the contamination of any CNSP. Purified Water (see Water for Pharmaceutical Purposes (1231)), distilled water, or reverse osmosis water should be used for rinsing equipment and utensils.

5. CLEANING AND SANITIZING

Cleaning and sanitizing of the surfaces in the nonsterile compounding area(s) must occur on a regular basis at the minimum frequencies specified in Table 1 or, if compounding is not performed daily, cleaning and sanitizing must be completed before initiating compounding. Cleaning and sanitizing must be repeated when spills occur and when surfaces are visibly soiled.
Cleaning and sanitizing agents must be selected and used with consideration of compatibilities, effectiveness, and to minimize the potential to leave residues.

If cleaning and sanitizing are performed as separate steps, cleaning must be performed first.

### Table 1. Minimum Frequency for Cleaning and Sanitizing Surfaces in Nonsterile Compounding Area(s)

<table>
<thead>
<tr>
<th>Site</th>
<th>Minimum Frequency</th>
</tr>
</thead>
</table>
| Work surfaces             | • At the beginning and end of each shift, after spills, and when surface contamination is known or suspected  
                           | • Clean and sanitize the work surfaces between compounding CNSPs with different components |
| Floors                    | Daily; after spills, and when surface contamination (e.g., splashes) is known or suspected |
| Walls                     | Every 3 months; after spills, and when surface contamination (e.g., splashes) is known or suspected |
| Ceilings                  | When visibly soiled and when surface contamination is known or suspected           |
| Storage shelving          | Every 3 months; after spills, and when surface contamination (e.g., splashes) is known or suspected |

### 6. EQUIPMENT AND COMPONENTS

#### 6.1 Equipment

The equipment and supplies used for compounding a CNSP must be suitable for the specific compounding process. Equipment surfaces that contact components must not be reactive, additive, or sorptive, and must not alter the quality of the CNSPs. Disposable or dedicated equipment may be used to reduce the chance of bioburden and cross-contamination.

Equipment must be stored in a manner to minimize the risk of contamination and must be located to facilitate its use, maintenance, and cleaning. Equipment and devices used in the compounding or testing of compounded preparations must be inspected prior to use and, if appropriate, verified for accuracy as recommended by the manufacturer and at the frequency recommended by the manufacturer, or at least every 12 months, whichever is more frequent. After compounding, the equipment must be cleaned to prevent cross-contamination of the next preparation.

Weighing, measuring, or otherwise manipulating components that could generate airborne chemical particles [e.g., active pharmaceutical ingredients (APIs), added substances, conventionally manufactured products] must be assessed to determine if these activities must be performed in closed system processing device to reduce the potential exposure to personnel or contamination of the facility or CNSPs. Examples of closed system processing devices include containment ventilated enclosures (CVEs), biological safety cabinets (BSCs), or single-use containment glove bags. The process evaluation must be carried out in accordance with the facility SOP and the assessment must be documented.

If a BSC or CVE is used, it must be certified every 12 months according to requirements such as the current Controlled Environment Testing Association (CETA), NSF International, or American Society of Heating, Refrigerating, and Air-Conditioning Engineers (ASHRAE) guidelines, or other laws and regulations of the applicable regulatory jurisdiction.

If a CVE or other non-disposable device is used, it must be cleaned as described in Table 2.

### Table 2. Minimum Frequency for Cleaning and Sanitizing Equipment in Nonsterile Compounding Area(s)

<table>
<thead>
<tr>
<th>Site</th>
<th>Minimum Frequency</th>
</tr>
</thead>
</table>
| CVE                               | • At the beginning and end of each shift, after spills, and when surface contamination is known or suspected  
                           | • Clean and sanitize the horizontal work surface of the CVE between compounding CNSPs with different components |
| Other devices and equipment used in compounding operations | • Before first use and thereafter in accordance with the manufacturer’s recommendations  
                           | • If no recommendation is available, after compounding CNSPs with different components |

#### 6.2 Components

The compounding facility must have written SOPs for the selection and inventory control of all components from receipt to use in a CNSP.

SDSs must be readily accessible to all personnel working with APIs and added substances located in the compounding facility. Personnel must be instructed on how to retrieve and interpret needed information.

**COMPONENT SELECTION**

A designated person must be responsible for selecting components to be used in compounding.

**APIs:**

- Must comply with the criteria in the USP–NF monograph, if one exists
COMPONENT RECEIPT

Upon receipt of components other than conventionally manufactured products, the COA must be reviewed to ensure that the component has met the acceptance criteria in a USP–NF monograph, if one exists. For components other than conventionally manufactured products, information including the receipt date, quantity received, supplier name, lot number, expiration date, and results of any in-house or third-party testing performed must be documented.

The date of receipt by the compounding facility must be clearly and indelibly marked on each component package that lacks a vendor expiration date. Packages of components (i.e., API and added substances) that lack a vendor’s expiration date must not be used by the compounding facility after 3 years from the date of receipt. A shorter expiration date must be assigned according to Pharmaceutical Compounding—Sterile Preparations (797), 9.3 Components, Component Receipt if the same component container is also used in sterile compounding or if the ingredient is known to be susceptible to degradation.

For each use, the lot must be examined for evidence of deterioration and other aspects of unacceptable quality. Once removed from the original container, components not used in compounding (e.g., excess after weighing) should be discarded and not returned to the original container to minimize the risk of contaminating the original container.

Before use, compounding personnel must visually re-inspect all components. Packages must be inspected to detect container breaks, looseness of the cap or closure, or deviation from the expected appearance or texture of the contents that might have occurred during storage.

Compounding personnel must ascertain before use that components are of the correct identity based on the labeling and have been stored under required conditions in the facility.

If the correct identity, strength, purity, and quality of components intended for preparation of CNSPs cannot be confirmed (e.g., containers with damaged or incomplete labeling), they must be immediately rejected. If they are not immediately discarded, they must be clearly labeled as rejected, and segregated to prevent their use before disposal.

COMPONENT EVALUATION BEFORE USE

All components must be handled in accordance with the manufacturer’s instructions or per laws and regulations of the applicable regulatory jurisdiction. The handling must minimize the risk of contamination, mix-ups, and deterioration (e.g., loss of identity, strength, purity, and quality).

COMPONENT SPILL AND DISPOSAL

The facility must maintain chemical hazard and disposal information (e.g., SDSs) and must review and update its chemical hazard and disposal information every 12 months. The chemical hazard and disposal information (e.g., SDSs) must be made accessible to compounding personnel.

The facility must have an SOP for the management of nonhazardous component spills and disposal. If required by the SOP, these activities must be documented and corrective action taken.

The facility must have a readily accessible spill kit in the compounding area. The contents of the spill kit should be affixed to the packaging of the spill kit if not readily visible on the manufacturer’s label.

All personnel who may be required to remediate a spill must receive training in spill management of chemicals used and stored at the compounding facility. Refresher training must be conducted every 12 months and documented for all personnel who may be required to clean up a spill.

Waste must be disposed of in accordance to laws and regulations of the applicable regulatory jurisdiction. The disposal of components must comply with laws and regulations of the applicable regulatory jurisdiction. For information on the handling of HDs, see (800).
7. MASTER FORMULATION AND COMPOUNDING RECORDS

7.1 Creating Master Formulation Records

A Master Formulation Record is a detailed record of procedures that describes how the CNSP is to be prepared. A Master Formulation Record must be created for each unique formulation of a CNSP. CNSPs are prepared according to the Master Formulation Record and the preparation information is documented on a Compounding Record (see 7.2 Creating Compounding Records). Any changes or alterations to the Master Formulation Record must be approved and documented according to the facility’s SOP. Box 7-1 lists the information that must be included in a Master Formulation Record:

Box 7-1. Master Formulation Records

A Master Formulation Record must include at least the following information:

- Name, strength or activity, and dosage form of the CNSP
- Identities and amounts of all components
  - If applicable, relevant characteristics of components (e.g., particle size, salt form, purity grade, solubility)
- Container–closure system(s)
- Complete instructions for preparing the CNSP, including equipment, supplies, and a description of the compounding steps
- Physical description of the final CNSP
- Assigned beyond-use date (BUD) and storage requirements
- Reference source to support the assigned BUD and storage requirements
- If applicable, calculations to determine and verify quantities and/or concentrations of components and strength or activity of API
- Labeling requirements (e.g., shake well)
- Quality control (QC) procedures (e.g., pH testing, visual inspection) and expected results
- Other information needed to describe the compounding process and ensure repeatability (e.g., adjusting pH, temperature)

7.2 Creating Compounding Records

A Compounding Record documents the compounding of each CNSP. A Compounding Record must be created for all CNSPs. Each Compounding Record must be reviewed for completeness before the CNSP is released. The identifier of the person completing the review and the date of review must be documented on the Compounding Record. The Compounding Record must permit traceability of all components in the case of a recall or known quality issue. The Master Formulation Record can be used as the basis for preparing the Compounding Record. For example, a copy of the Master Formulation Record can be made that contains spaces to record the information needed to complete the Compounding Record. Box 7-2 lists the information that must be included in a Compounding Record:

Box 7-2. Compounding Records

Compounding Records must include at least the following information:

- Name, strength or activity, and dosage form of the CNSP
- Date and time of preparation of the CNSP
- Assigned internal identification number (e.g., prescription, order, or lot number)
- A method to identify the individuals involved in the compounding process and verifying the final CNSP
- Name, vendor or manufacturer, lot number, and expiration date of each component
- Weight or measurement of each component
- Total quantity compounded
- Assigned BUD and storage requirements
- If applicable, calculations to determine and verify quantities and/or concentrations of components and strength or activity of API
- Physical description of the final CNSP
- Results of quality control procedures (e.g., pH testing, visual inspection)
- Master Formulation Record reference for the CNSP

8. RELEASE INSPECTIONS

At the completion of compounding and before release and dispensing, the CNSP must be visually inspected to determine whether the physical appearance is as expected. Inspections must also confirm that the CNSP and its labeling match the Compounding Record and the prescription or medication order. Some CNSPs, as noted in their Master Formulation Record, also must be visually checked for certain characteristics (e.g., emulsions must be checked for phase separation). All checks and inspections, and if required, any other tests necessary to ensure the quality of the CNSP must be detailed in the facility’s Master Formulation Records. Checks and inspections must be documented. Additional quality assurance (QA) and quality control activities are described in 12. Quality Assurance and Quality Control. Pre-release inspection also must include a visual inspection of container–closure integrity (e.g., checking for leakage, cracks in the container, or improper seals). CNSPs with observed defects must be immediately discarded, or marked and segregated from acceptable units in a manner that prevents them from being released or dispensed.
9. LABELING

The term labeling designates all labels and other written, printed, or graphic matter on the immediate container or on, or in, any package or wrapper in which the article is enclosed, except any outer shipping container. The term label designates the part of the labeling on the immediate container. See Labeling (7).

Every dispensed CNSP must be labeled with adequate, legible identifying information to prevent errors during storage, dispensing, and use. All labeling must be in compliance with laws and regulations of the applicable regulatory jurisdiction. The label on each immediate container of the CNSP must, at a minimum, display the following information:

- Assigned internal identification number (e.g., barcode, prescription, order, or lot number)
- Active component(s), and amounts, activities, or concentrations
- Dosage form
- Amount or volume in each container
- Storage conditions if other than controlled room temperature
- BUD

The labeling on the CNSP should display the following information:

- Route of administration
- Indication that the preparation is compounded
- Any special handling instructions
- Any warning statements that are applicable
- Name, address, and contact information of the compounding facility if the CNSP is to be sent outside of the facility or healthcare system in which it was compounded

Labeling operations must be controlled to prevent labeling errors and CNSP mix-ups. A final check must be conducted to verify that the correct label has been affixed to the finished CNSP. All labels must also comply with laws and regulations of the applicable regulatory jurisdiction.

10. ESTABLISHING BEYOND-USE DATES

10.1 Terminology

Each CNSP label must state the date, or the hour and date, beyond which the preparation cannot be used and must be discarded (i.e., the BUD). BUDs for CNSPs are calculated in terms of hours, days, or months.

BUDs and expiration dates are not the same. An expiration date identifies the time during which a conventionally manufactured drug product, active ingredient, or added substance can be expected to meet the requirements of a compendial monograph, if one exists, or maintain expected quality provided it is kept under the specified storage conditions. The expiration date limits the time during which a conventionally manufactured product, API, or added substance may be dispensed or used (see Labeling (7), Labels and Labeling for Products in Other Categories, Expiration Date and Beyond-Use Date). Expiration dates are assigned by manufacturers based on analytical and performance testing of the sterility, chemical and physical stability, and packaging integrity of the product. Expiration dates are specific for a particular formulation in its container and at stated exposure conditions of illumination and temperature.

10.2 Parameters to Consider in Establishing a BUD

BUDs for CNSPs should be established conservatively to ensure that the preparation maintains its required characteristics to minimize the risk of contamination or degradation.

When establishing a BUD for a CNSP, it is critical that personnel carefully consider the possible ways that the physical or chemical characteristics of the CNSP could change over time. The following factors must be considered:

- The chemical and physical stability properties of the API and any added substances in the preparation (e.g., if the API and added substances in the preparation are known to degrade over time and/or under certain storage conditions, which would reduce the strength of the preparation and/or produce harmful impurities)
- The compatibility of the container–closure system with the finished preparation (e.g., leachables, interactions, adsorption, and storage conditions)
- Degradation of the container–closure system, which can lead to a reduction in integrity of the CNSP
- The potential for microbial proliferation in the CNSP

10.3 Establishing a BUD for a CNSP

The BUDs indicate the days after the CNSP is prepared and beyond which the CNSP must not be used. The day that the preparation is compounded is considered Day 1. The BUDs in Table 3 are based on the ability of the CNSP to maintain chemical and physical stability and to suppress microbial growth. Table 3 represents the maximum BUDs for CNSPs that are packaged in tight, light-resistant containers unless conditions under 10.4 CNSPs Requiring Shorter BUDs or 10.5 Extending BUDs for CNSPs apply.
The aqueous and nonaqueous dosage forms in Table 3 are defined based on the water activity (Aw) of the most similar drug product described in Application of Water Activity Determination to Nonsterile Pharmaceutical Products (1112). In general, the use of Aw aids in assessing the susceptibility of CNSPs to microbial contamination and the potential for API degradation due to hydrolysis. Reduced Aw greatly assists in the prevention of microbial proliferation in conventionally manufactured products and is expected to convey the same benefit to CNSPs. The list of manufactured products in Application of Water Activity Determination to Nonsterile Pharmaceutical Products (1112), Table 2 is not exhaustive. However, it provides guidance on the Aw value of a particular CNSP and can assist personnel in determining the BUD by dosage form based on Table 3.

CNSPs with an Aw > 0.6 should contain suitable antimicrobial agents to protect against bacteria, yeast, and mold contamination from proliferation if inadvertently introduced during or after the compounding process. When antimicrobial preservatives are clinically contraindicated in a CNSP, storage of the preparation in a refrigerator is required if such storage does not change the physical or chemical properties of the CNSP (i.e., precipitation).

### Table 3. Maximum BUD by Type of Preparation in the Absence of a USP–NF Compounded Preparation Monograph or CNSP-Specific Stability Information

<table>
<thead>
<tr>
<th>Type of Preparation</th>
<th>BUDs (days)</th>
<th>Storage Temperature*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-preserved aqueous dosage forms</td>
<td>14</td>
<td>Refrigerator</td>
</tr>
<tr>
<td>Preserved aqueous dosage forms</td>
<td>35</td>
<td>Controlled room temperature</td>
</tr>
<tr>
<td>Nonaqueous dosage forms</td>
<td>90</td>
<td>Controlled room temperature</td>
</tr>
<tr>
<td>Solid dosage forms</td>
<td>180</td>
<td>Controlled room temperature</td>
</tr>
</tbody>
</table>

*See Packaging and Storage Requirements (659).

An aqueous preparation is one that has an Aw of > 0.6 (e.g., emulsions, gels, creams, solutions, sprays, or suspensions).

Any preparation other than solid dosage forms that have a reduced Aw of ≤0.6 (e.g., suppositories, ointments, fixed oils, or waxes).

Capsules, tablets, granules, powders.

### 10.4 CNSPs Requiring Shorter BUDs

A shorter BUD must be established under the following circumstances:

- If the API or any other components in the CNSP have an expiration date that is earlier than the BUD that could be assigned from Table 3, the expiration date supersedes the BUD and must be the assigned shortest date.
- If the CNSP includes components from conventionally manufactured product(s), the BUD of the CNSP must not exceed the shortest remaining expiration date of any of those conventionally manufactured product(s).
- If the CNSP includes components from other compounded preparations, the BUD of the final CNSP must not exceed the shortest remaining BUD of any of those compounded preparations.
- If the formulation is known to require a shorter BUD.

### 10.5 Extending BUDs for CNSPs

#### CNSPs with a USP–NF Monograph

If there is a USP–NF compounded preparation monograph for the CNSP, the BUD must not exceed the BUD specified in the monograph.

#### CNSPs with Stability Information

The BUDs specified in Table 3 for aqueous dosage forms and nonaqueous dosage forms may be extended up to maximum of 180 days if there is a stability study (published or unpublished) using a stability-indicating assay for the API(s), CNSP, and type of container–closure that will be used. If the BUD of the CNSP is extended beyond the BUDs in Table 3, an aqueous CNSP should be tested for antimicrobial effectiveness (see Antimicrobial Effectiveness Testing (S1)). The compounder may rely on 1) antimicrobial effectiveness testing that is conducted (or contracted for) once for each formulation in the particular container–closure system in which it will be packaged or 2) antimicrobial effectiveness testing results provided by an FDA-registered facility or published in peer-reviewed literature sources if the CNSP formulation (including any preservative) and container–closure system are exactly the same as those tested unless a bracketing study is performed. Antimicrobial effectiveness testing may be performed on a low concentration and a high concentration of the active ingredient in the formulation to establish preservative effectiveness across various strengths of the same formulation (e.g., bracketing). The concentration of all other ingredients (including preservatives) must be the same throughout the bracketing study.

### 11. SOPS

Facilities preparing CNSPs must develop SOPs on all aspects of the compounding operation. All personnel who conduct or oversee compounding activities must be trained in the SOPs and are responsible for ensuring that they are followed.
One or more person(s) must be designated to ensure that SOPs are fully implemented. The designated person(s) must ensure that follow-up occurs if problems, deviations, or errors are identified.

12. QUALITY ASSURANCE AND QUALITY CONTROL

Quality assurance and quality control programs are necessary to ensure that consistently high-quality CNSPs are prepared. QA is a system of procedures, activities, and oversight that ensures that the compounding process consistently meets quality standards. QC is the sampling, testing, and documentation of results that, taken together, ensure that specifications have been met before release of the CNSP. See Quality Assurance in Pharmaceutical Compounding (1163).

A facility’s QA and QC programs must be formally established and documented in SOPs that ensure that all aspects of the preparation of CNSPs are conducted in accordance with this chapter and laws and regulations of the applicable regulatory jurisdiction. A designated person must ensure that the facility has formal, written QA and QC programs that establish a system of:
1. Adherence to procedures
2. Prevention and detection of errors and other quality problems
3. Evaluation of complaints and adverse events
4. Appropriate investigations and corrective actions

The SOPs must describe the roles, duties, and training of the personnel responsible for each aspect of the QA program. Designated person(s) responsible for the QA program must have the training, experience, responsibility, and authority to perform these duties. The overall QA and QC program must be reviewed at least once every 12 months by the designated person(s). The results of the review must be documented and appropriate action must be taken if needed.

13. CNSP PACKAGING AND TRANSPORTING

13.1 Packaging of CNSPs

SOPs must describe packaging of CNSPs. Personnel should select and use packaging materials that will maintain the physical and chemical integrity and stability of the CNSPs. Packaging materials must protect CNSPs from damage, leakage, contamination, and degradation, while simultaneously protecting personnel from exposure.

13.2 Transporting CNSPs

If transporting CNSPs, the facility must have written SOPs to describe the mode of transportation, any special handling instructions, and whether temperature monitoring devices are needed.

14. COMPLAINT HANDLING AND ADVERSE EVENT REPORTING

Compounding facilities must develop and implement SOPs for complaint and adverse event report receipt, acknowledgment, and handling and designate one or more person(s) to be responsible for handling them. Complaints may include concerns or reports on the quality and labeling of, or possible adverse reactions to, a specific CNSP.

14.1 Complaint Handling

The designated person(s) must ensure that all complaints are reviewed to determine whether the complaint indicates a potential quality problem with the CNSP. If it does, a thorough investigation into the cause of the problem must be initiated and completed. The investigation must consider whether the quality problem extends to other CNSPs. Corrective action, if necessary, must be implemented for all potentially affected CNSPs. Consider whether to initiate a recall of potentially affected CNSPs and whether to cease nonsterile compounding processes until all underlying problems have been identified and corrected.

A readily retrievable written or electronic record of each complaint must be kept by the facility, regardless of the source of the complaint (e.g., e-mail, telephone, mail). The record must contain the name of the complainant or unique identifier, the date the complaint was received, the nature of the complaint, and the response to the complaint. In addition, to the extent that the information is known, the following should be recorded: the name and strength of the CNSP, the prescription or medication order number, and the lot number, if one is assigned.

The record must also include the findings of any investigation and any follow-up. Records of complaints must be easily retrievable for review and evaluation for possible trends and must be retained in accordance with the record-keeping requirements in 15. Documentation. A CNSP that is returned in connection with a complaint must be quarantined until it is destroyed after completion of the investigation and in accordance with laws and regulations of the applicable regulatory jurisdiction.
14.2 Adverse Event Reporting

The designated person(s) must ensure that reports of potential adverse events involving a CNSP are reviewed. If the investigation into an adverse event reveals a quality problem with a CNSP that is likely to affect other patients, those patients and prescribers potentially affected must be informed. The designated person(s) must review all adverse event reports as part of the QA and QC programs (see 12. Quality Assurance and Quality Control). Adverse events must be reported in accordance with facility SOPs and all laws and regulations of the applicable regulatory jurisdiction. In addition, adverse events associated with a CNSP should be reported to the FDA through the MedWatch program for human drugs and through Form FDA 1932a for animal drugs.

15. DOCUMENTATION

All facilities where CNSPs are prepared must have and maintain written or electronic documentation to demonstrate compliance with the requirements in this chapter. This documentation must include, but is not limited to, the following:

- Personnel training, competency assessments, and corrective actions for any failures
- Equipment records (e.g., calibration, verification, and maintenance reports)
- COA
- Receipt of components
- SOPs, Master Formulation Records, and Compounding Records
- Release inspection and testing records
- Information related to complaints and adverse events including corrective actions taken
- Results of investigation and corrective actions

Documentation must comply with all laws and regulations of the applicable regulatory jurisdiction. Records must be legible and stored in a manner that prevents their deterioration and/or loss. All required compounding records for a particular CNSP (e.g., Master Formulation Record, Compounding Record, and release inspection and testing results) must be readily retrievable for at least 3 years after preparation or as required by the laws and regulations of the applicable regulatory jurisdiction, whichever is longer.

GLOSSARY

Active pharmaceutical ingredient (API): Any substance or mixture of substances intended to be used in the compounding of a preparation, thereby becoming the active ingredient in that preparation and furnishing pharmacological activity or other direct effect in the diagnosis, cure, mitigation, treatment, or prevention of disease in humans and animals or affecting the structure and function of the body.

Added substances: Ingredients that are necessary to compound a preparation but are not intended or expected to cause a pharmacologic response if administered alone in the amount or concentration contained in a single dose of the compounded preparation. The term is used synonymously with the terms inactive ingredients, excipients, and pharmaceutical ingredients.

Biological safety cabinet (BSC): A ventilated cabinet which may be used for compounding. These cabinets divided into three general classes (Class I, Class II, and Class III). Class II BSCs are further divided into types (Type A1, Type A2, Type B1, and Type B2).

Certificate of Analysis (COA): A report from the supplier of a component, container, or closure that accompanies the supplier’s material and contains the specifications and results of all analyses and a description of the material.

Cleaning: The process of removing soil (e.g., organic and inorganic material) from objects and surfaces, normally accomplished by manually or mechanically using water with detergents or enzymatic products.

Component: Any ingredient used in the compounding of a preparation, including any active ingredient, added substance, or conventionally manufactured product.

Compounded nonsterile preparation (CNSP): A preparation intended to be nonsterile created by combining admixing, diluting, pooling, reconstituting other than as provided in the manufacturer’s labeling, or otherwise altering of a drug or bulk drug substance.

Compounder: Personnel trained to compound preparations.

Compounding: The process of combining, admixing, diluting, pooling, reconstituting other than as provided in the manufacturer’s labeling, or otherwise altering a drug or bulk drug substance to create a nonsterile medication.

Compounding area: A space that is specifically designated for nonsterile compounding. A visible perimeter should establish the boundaries of the nonsterile compounding area.

Container–closure system: Packaging components that together contain and protect the dosage form. This includes primary packaging components and secondary packaging components, if the latter are intended to provide additional protection.

Containment glove bag: A single-use disposable glove bag that is capable of containing airborne chemical particles.

Containment ventilated enclosure (CVE): A full or partial enclosure that uses ventilation principles to capture, contain, and remove airborne contaminants through high-efficiency particulate air (HEPA) filtration and to prevent their release into the work environment.

Conventionally manufactured product: A pharmaceutical dosage form, usually the subject of an FDA-approved application that is manufactured under current good manufacturing practice conditions.

Designated person(s): One or more individuals assigned to be responsible and accountable for the performance and operation of the facility and personnel for the preparation of CNSPs.
Hazardous drug (HD): Any drug identified by at least one of the following six criteria: carcinogenicity, teratogenicity or developmental toxicity, reproductive toxicity in humans, organ toxicity at low dose in humans or animals, genotoxicity, or new drugs that mimic existing HDs in structure or toxicity. See (800).

Label: A display of written, printed, or graphic matter on the immediate container of any article.

Labeling: All labels and other written, printed, or graphic matter that are 1) on any article or any of its containers or wrappers, or 2) accompanying such an article.

Purified Water: The minimal quality of source water for the production of Purified Water is drinking water whose attributes are prescribed by the US Environmental Protection Agency (EPA), the EU, Japan, or the World Health Organization (WHO). This source water may be purified using unit operations that include deionization, distillation, ion exchange, reverse osmosis, filtration, or other suitable purification procedures. (See Water for Pharmaceutical Purposes (1231), 3. Waters Used for Pharmaceutical Manufacturing and Testing Purposes, 3.1 Bulk Monographed Waters and Steam, 3.1.1 Purified Water.)

Preservative: A substance added to inhibit microbial growth.

Quality assurance (QA): A system of procedures, activities, and oversight that ensures that the compounding process consistently meets quality standards.

Quality control (QC): The sampling, testing, and documentation of results that, taken together, ensure that specifications have been met before release of the CNSP.

Reconstitution: The process of adding a diluent to a conventionally manufactured product to prepare a solution or suspension.

Release inspection and testing: Visual inspection and testing performed to ensure that a preparation meets appropriate quality characteristics.

Sanitizing agent: An agent for reducing, on inanimate surfaces, the number of all forms of microbial life including fungi, viruses, and bacteria.

Specification: The tests, analytical methods, and acceptance criteria to which an API or other components, CNSP, container–closure system, equipment, or other material used in compounding CNSPs must conform to be considered acceptable for its intended use.

Stability: The extent to which a product or preparation retains physical and chemical properties and characteristics within specified limits throughout its expiration or BUD.

### APPENDIX

<table>
<thead>
<tr>
<th>Acronyms</th>
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<tbody>
<tr>
<td>API(s)</td>
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<tr>
<td>ASHRAE</td>
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<tr>
<td>Aw</td>
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<tr>
<td>BSC(s)</td>
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<tr>
<td>BUD(s)</td>
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<tr>
<td>CETA</td>
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<tr>
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<td>SDS(s)</td>
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<td>SOP(s)</td>
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</table>

### POLAROGRAPHY

Polarography is an electrochemical method of analysis based on the measurement of the current flow resulting from the electrolysis of a solution at a polarizable microelectrode, as a function of an applied voltage. The polarogram (see Figure 1) obtained by this measurement provides qualitative and quantitative information on electro-reducible and electro-oxidizable substances. The normal concentration range for substances being analyzed is from $10^{-2}$ molar to $10^{-5}$ molar.

In direct current (dc) polarography, the microelectrode is a dropping mercury electrode (DME) consisting of small reproducible drops of mercury flowing from the orifice of a capillary tube connected to a mercury reservoir. A saturated calomel electrode (SCE) with a large surface area is the most commonly employed reference electrode. As the voltage applied to the cell increases, only a very small residual current flows until the substance under assay undergoes reduction or oxidation. Then
the current increases, at first gradually, then almost linearly with voltage, and it gradually reaches a limiting value as is shown in Figure 1.

![Typical Polarogram Showing Change in Current Flow with Increasing Potential Applied to the Dropping Mercury Electrode.](image)

Fig. 1. Typical Polarogram Showing Change in Current Flow with Increasing Potential Applied to the Dropping Mercury Electrode.

On the initial rising portion of the polarographic wave, the increased flow of current results in a decrease in the concentration of the electro-active species at the electrode surface. As the voltage and current increase, the concentration of the reactive species decreases further to a minimal value at the electrode surface. The current is then limited by the rate at which the reacting species can diffuse from the bulk of the solution to the surface of the microelectrode. The final current rise is caused by the reaction of the supporting electrolyte. This large concentration of electrolyte is inert within the potential range used in the analysis, and it prevents the reactive species from reaching the electrode by electrical migration, thus assuring that the limiting current is diffusion-controlled.

Since, in the case of the DME, the electrode surface is being constantly renewed in a cyclic fashion, the current increases from a small value as the drop begins to form to a maximum value as the drop falls. By the use of a suitable recorder to measure the current, the characteristic saw-toothed record is obtained. The limiting current is the sum of the residual and the diffusion currents. The residual current is subtracted from the limiting current to give the wave height.

**ILKOVIC EQUATION**

The linear relationship between the diffusion current \( i_d \) and the concentration of electro-active species is shown by the Ilkovic equation:

\[
i_d = 708nD^{1/2}Cm^{2/3}t^{1/6}
\]

in which \( i_d \) is the maximum current in microamperes; \( n \) is the number of electrons required per molecule of electro-active substance; \( D \) is its diffusion coefficient, in square cm per second; \( C \) is the concentration, in millimoles per L; \( m \) is the rate of mercury flow from the DME, in mg per second; and \( t \) is the drop time, in seconds.

Modern polarographs are equipped with recorders capable of following the current during the latter portion of the drop life; consequently, the maximum of the oscillations is the measure of the current. When the current is measured only at the end of the drop life, the technique is termed sampled dc polarography. In this case, only the maximum currents are recorded and oscillations due to drop growth are not observed.

For instruments equipped with galvanometers to measure the current or recorders operated in a damped mode, the saw-toothed waves correspond to oscillations about the average current. In the latter case, the average of the oscillations is the measure of the current. For polarograms obtained in this manner, the \( i_d \), given by the Ilkovic equation is the average current in microamperes observed during the life of the drop, when the coefficient 708 is replaced by 607.
CONTROL OF THE DIFFUSION CURRENT

The Ilkovic equation identifies the variables that must be controlled to ensure that the diffusion current is directly proportional to the concentration of electro-active material. At 25°C the diffusion coefficients for aqueous solutions of many ions and organic molecules increase 1% to 2% per degree rise in temperature. Thus the temperature of the polarographic cell must be controlled to within ±0.5°C. The quantities m and t depend upon the dimensions of the capillary and the height of the mercury column above the electrode. Although results obtained with different capillaries can be compared if the product m²t₁⁸ is known, it is advisable to use the same capillary with a constant head of mercury during a series of analyses. The diffusion current is proportional to the square root of the height of the mercury column. A mercury reservoir with a diameter greater than 4 cm prevents any significant drop in the mercury level during a series of runs.

The capillary for the DME has a bore of approximately 0.04 mm and a length of 6 cm to 15 cm. The height of the mercury column, measured from the tip of the capillary to the top of the mercury pool, ranges from 40 cm to 80 cm. The exact length of the capillary and the height of the mercury column are adjusted to give a drop-time of between 3 and 5 seconds at open circuit with the capillary immersed in the test solution.

Equipment is available that allows controlled drop-times of fractions of a second to several seconds. As detail within a polarogram is related to the number of drops delivered during a given potential change, such short drop-times allow more rapid recording of the polarogram.

The current flowing through the test solution during the recording of a polarogram is in the microampere range. Thus, the current produces negligible changes in the test solution and several polarograms can be run on the same test solution without significant differences.

HALF-WAVE POTENTIAL

The half-wave potential (E½) occurs at the point on the polarogram one-half the distance between the residual current and the limiting current plateau. This potential is characteristic of the electro-active species and is largely independent of its concentration or the capillary used to obtain the wave. It is dependent upon the solution composition and may change with variations in the pH or in the solvent system or with the addition of complexing agents. The half-wave potential thus serves as a criterion for the qualitative identification of a substance.

The potential of the DME is equal to the applied voltage versus the reference electrode after correction for the iR drop (that potential need to pass the current, i, through the solution with a resistance R). It is especially important to make this correction for nonaqueous solutions, which ordinarily possess high resistance, if an accurate potential for the DME is needed. Correction of the half-wave potential is not required for quantitative analysis. Unless otherwise indicated, it is to be understood that potentials represent measurements made against the SCE.

REMOVAL OF DISSOLVED OXYGEN

Inasmuch as oxygen is reduced at the DME in two steps, first to hydrogen peroxide and then to water, it interferes where polarograms are to be made at potentials more negative than about 0 volt versus SCE, and must be removed. This may be accomplished by bubbling oxygen-free nitrogen through the solution for 10 to 15 minutes immediately before recording the wave, the nitrogen first having been “conditioned” to minimize changes due to evaporation, by being passed through a separate portion of the solution.

It is necessary that the solution be quiet and vibration-free during the time the wave is recorded, to ensure that the current is diffusion-controlled. Therefore, the nitrogen aeration should be stopped and the gas be directed to flow over the surface of the solution before a polarogram is recorded.

In alkaline media, sodium bisulfite may be added to remove oxygen, provided the reagent does not react with other components of the system.

MEASUREMENT OF WAVE HEIGHT

To use a polarogram quantitatively, it is necessary to measure the height of the wave. Since this is a measure of the magnitude of the diffusion current, it is measured vertically. To compensate for the residual current, the segment of the curve preceding the wave is extrapolated beyond the rise in the wave. For a well-formed wave where this extrapolation parallels the limiting current plateau, the measurement is unambiguous. For less well-defined waves, the following procedure may be used unless otherwise directed in the individual monograph. Both the residual current and the limiting current are extrapolated with straight lines, as shown by the graph (Figure 1). The wave height is taken as the vertical distance between these lines measured at the half-wave potential.

PROCEDURE

[CAUTION—Mercury vapor is poisonous, and metallic mercury has a significant vapor pressure at room temperature. The work area in which mercury is used should be constructed in such a way that any spilled or spattered droplets of mercury can be completely recovered with relative ease. Scrupulously clean up mercury after each use of the instrument. Work in a well-ventilated laboratory, taking care to clean up any spilled mercury.]

Transfer a portion of the final dilution of the substance being assayed to a suitable polarographic cell immersed in a water bath regulated to 25 ± 0.5°C. Pass a stream of nitrogen through the solution for 10 to 15 minutes to remove dissolved oxygen.

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Start the mercury dropping from the capillary, insert the capillary into the test solution, and adjust the height of the mercury reservoir. Switch the flow of nitrogen to pass over the surface of the solution, and record the polarogram over the potential range indicated in the individual monograph, using the appropriate recorder or galvanometer sensitivity to give a suitable wave. Measure the height of the wave, and unless otherwise directed in the monograph, compare this with the wave height obtained with the appropriate USP Reference Standard, measured under the same conditions.

**PULSE POLAROGRAPHY**

In conventional dc polarography, the current is measured continuously as potential is applied as a linear ramp (see *Figure 2*).

![Fig. 2. Direct Current (dc) Polarography.](image)

This current is composed of two elements. The first, the diffusion (faradaic) current, is produced by the substance undergoing reduction or oxidation at the working electrode, and is directly proportional to the concentration of this substance. The second element is the capacitative current (charging of the electrochemical double layer). The changes in these currents as the mercury drop varies in size produce the oscillations present in typical dc polargrams.

In normal pulse polarography, a potential pulse is applied to the mercury electrode near the end of the drop life, with the drop being held at the initial potential during growth period (see *Figure 3*).

![Fig. 3. Pulse Polarography.](image)

Each succeeding drop has a slightly higher pulse applied to it, with the rate of increase being determined by the selected scan rate. The current is measured at the end of the pulse where the capacitative current is nearly zero, and thus primarily faradaic current is measured (see *Figure 4*).
In addition, since the pulse is applied for only a short duration, the diffusion layer is not depleted as extensively as in dc polarography and larger current levels are obtained for equivalent concentrations. Concentrations as low as $10^{-6}$ M can be measured, providing approximately a ten-fold increase in sensitivity over that with dc polarography. Limiting current values are more easily measured, since the waves are free from oscillations.

Differential pulse polarography is a technique whereby a fixed-height pulse applied at the end of the life of each drop is superimposed on a linear increasing dc ramp (see Figure 5).

Current flow is measured just before application of the pulse and again at the end of the pulse. The difference between these two currents is measured and presented to the recorder. Such a differential signal provides a curve approximating the derivative of the polarographic wave, and gives a peak presentation. The peak potential is equivalent to:

$$E_{1/2} - \Delta E/2$$

where $\Delta E$ is the pulse height. The peak height is directly proportional to concentration at constant scan rates and constant pulse heights. This technique is especially sensitive (levels of $10^{-7}$ M may be determined) and affords improved resolution between closely spaced waves.

**ANODIC STRIPPING VOLTAMMETRY**

Anodic stripping voltammetry is an electrochemical technique whereby trace amounts of substances in solution are concentrated (reduced) onto an electrode and then stripped (oxidized) back into solution by scanning the applied voltage anodically. The measurement of the current flow as a function of this voltage and scanning rate provides qualitative and quantitative information on such substances. The concentration step permits analyses at $10^{-7}$ M to $10^{-9}$ M levels.
Basic instrumentation includes a voltage ramp generator; current-measuring circuitry; a cell with working, reference, and counter electrodes; and a recorder or other read-out device. Instruments having dc or pulse-polarographic capabilities are generally quite adequate for stripping application. The working electrode commonly used is the hanging mercury drop electrode (HMDE), although the mercury thin-film electrode (MTFE) has acquired acceptance. For analysis of metals such as silver, platinum, and gold, whose oxidation potentials are more positive than mercury, and mercury itself, the use of solid electrodes such as platinum, gold, or carbon is required. A saturated calomel electrode or a silver–silver chloride electrode serves as the reference except for the analysis of mercury or silver. A platinum wire is commonly employed as the counter electrode.

Test specimens containing suitable electrolyte are pipetted into the cell. Dissolved oxygen is removed by bubbling nitrogen through the cell for 5 to 10 minutes.

Generally, an electrolysis potential equivalent to 200 to 300 mV more negative than the half-wave potential of the material to be analyzed is applied (although this potential is to be determined experimentally), with stirring for 1 to 10 minutes. For reproducible results, maintain constant conditions (i.e., deposition time, stirring rate, temperature, specimen volume, and drop size if HMDE is used).

After deposition, the stirring is discontinued and the solution and electrode are allowed to equilibrate for a short period. The potential is then rapidly scanned anodically (10 mV/second or greater in dc polarography and 5 mV/second in differential pulse polarography). As in polarography, the limiting current is proportional to concentration of the species (wave height in dc and pulse; peak height in differential pulse), while the half-wave potential (dc, pulse) or peak potential (differential pulse) identifies the species. It is imperative that the choice of supporting electrolyte be made carefully in order to obtain satisfactory behavior. Quantitation is usually achieved by a standard addition or calibration method.

This technique is appropriate for trace-metal analysis, but has limited use in organic determinations, since many of these reactions are irreversible. In analyzing substances such as chloride, cathodic stripping voltammetry may be used. The technique is the same as anodic stripping voltammetry, except that the substance is deposited anodically and then stripped by a cathodic voltage scan.

### 〈831〉 REFRACTIVE INDEX

The refractive index \( n \) of a substance is the ratio of the velocity of light in air to the velocity of light in the substance. It is valuable in the identification of substances and the detection of impurities.

Although the standard temperature for Pharmacopeial measurements is 25°, many of the refractive index specifications in the individual monographs call for determining this value at 20°. The temperature should be carefully adjusted and maintained, since the refractive index varies significantly with temperature.

The values for refractive index given in this Pharmacopeia are for the D line of sodium (doublet at 589.0 nm and 589.6 nm). Most instruments available are designed for use with white light but are calibrated to give the refractive index in terms of the D line of sodium light.

The Abbé refractometer measures the range of refractive index for those Pharmacopeial materials for which such values are given. Other refractometers of equal or greater accuracy may be employed.

To achieve the theoretical accuracy of ±0.0001, it is necessary to calibrate the instrument against a standard provided by the manufacturer and to check frequently the temperature control and cleanliness of the instrument by determining the refractive index of distilled water, which is 1.3330 at 20° and 1.3325 at 25°.

### 〈841〉 SPECIFIC GRAVITY

**Change to read:**

\( \Delta \) Specific gravity is the term for the relative density of a substance when water is used as the reference. The measurement of specific gravity necessarily involves the measurement of the density of the sample at a specified temperature as well as the measurement of the density of water.

The density \( \rho \) (g/mL or g/cm\(^3\)) means the mass per unit volume, and the relative density means the ratio of the mass of a sample specimen to that of an equal volume of a standard substance. The relative density and specific gravity are unitless values.

The specific gravity \( \left( d_2^\prime \right) \) means the ratio of the mass of the sample specimen at \( t^\prime \) to that of an equal volume of water (H\(_2\)O) at \( t \). \( \Delta \) Unless otherwise stated in the individual monograph, the specific gravity determination is applicable only to liquids and \( \Delta \) (USP 1-Aug-2020). Where a temperature is specified in the individual monograph, the specific gravity is based on the ratio of the weight of a liquid in air at 25° to that of an equal volume of water at the same temperature \( \Delta \) (i.e., \( d_{25}^\prime \)). \( \Delta \) (USP 1-Aug-2020) Where a temperature is specified in the individual monograph, the specific gravity is the ratio of the weight of the liquid in air at the specified temperature to that of an equal volume of water at the same temperature. \( \Delta \) (USP 1-Aug-2020) If the substance is a solid at 25°, determine the specific gravity \( \Delta \) above the melting point \( \Delta \) (USP 1-Aug-2020) (at the temperature directed in the individual monograph, \( \Delta \) if specified). \( \Delta \) (USP 1-Aug-2020)

Unless otherwise stated in the individual monograph, the density is defined as the mass of a unit volume of the substance at 25°, expressed in kilograms per cubic meter or grams per cubic centimeter \( (1 \text{ kg/m}^3 = 10^{-3} \text{ g/cm}^3) \). Where the density is known, mass can be converted to volume, or volume converted to mass, by \( \Delta \) using \( \Delta \) (USP 1-Aug-2020) the formula, volume = mass/density.

\( \Delta \) (USP 1-Aug-2020)
**METHOD I**

**Procedure**

Select a clean, dry pycnometer that has been previously calibrated by determining its weight and the weight of the deaerated water contained in it at 25°. Adjust the temperature of the sample liquid to about 20°, and fill the pycnometer with it. Adjust the temperature of the filled pycnometer to 25°, remove any excess liquid, and weigh. When the monograph specifies a temperature different from 25°, the filled pycnometers must be brought to the temperature of the balance before weighing. The specific gravity ($d_t'$) can be calculated by use of the following equation:

$$d_t' = \frac{W_t - W}{W_t - W_2}$$

where $W_t$ = mass of the pycnometer filled with the sample solution at $t'$

$W$ = mass of the clean, dry pycnometer

$W_2$ = mass of the pycnometer filled with water at $t$

The density of water can be used to calculate the density of the sample of the specimen according to the following equation:

$$\rho_{s,t'} = \frac{\rho_{w,t'}}{\rho_{w,t}}$$

$\rho_{s,t'}$ = density of the sample at $t'$

$\rho_{w,t}$ = density of water at $t$

**METHOD II**

The procedure requires the use of the oscillating transducer density meter. The apparatus consists of the following:

- A U-shaped tube, usually of borosilicate glass, which contains the liquid to be examined
- A magneto-electrical or piezo-electrical excitation system that causes the tube to oscillate as a cantilever oscillator at a characteristic frequency depending on the density of the liquid to be examined
- A means of measuring the oscillation period ($T$), which may be converted by the apparatus to give a direct reading of density or used to calculate density by using the constants $A$ and $B$ described below
- A means to measure and/or control the temperature of the oscillating transducer containing the liquid to be tested

The oscillation period is a function of the spring constant ($c$) and the mass of the system:

$$T^2 = \left(\frac{M}{c} + \left[\rho \times V/c\right]\right) \times 4\pi^2$$

where $\rho$ is the density of the liquid to be tested, $M$ is the mass of the tube, and $V$ is the volume of the filled tube.

Introduction of two constants $A = c/(4\pi^2 \times V)$ and $B = (M/V)$ leads to the classical equation for the oscillating transducer:

$$\rho = A \times T^2 - B$$

The specific gravity of the liquid is given by the formula:

$$\rho_{s}/\rho_{w}$$

where $\rho_{s}$ and $\rho_{w}$ are the densities of the liquid and water, respectively, both determined at 25°, unless otherwise directed in the individual monograph.

Samples with high viscosity will dampen the oscillations in the instrument and will introduce an error in the density measurement. Some oscillating transducer density meters are able to provide a correction to the density based on the viscosity of the sample. Samples with viscosities <10 mPa⋅s can be accurately measured without a density correction. Samples with viscosities >10 mPa⋅s must be measured with a density correction. If a density correction is not possible for a sample with >10 mPa⋅s viscosity, use Method I.

**Calibration**

The constants $A$ and $B$ are determined by operating the instrument with the U-shaped tube filled with two different samples of known density (e.g., degassed water and air). Perform the control measurements daily, using degassed water. The results displayed for the control measurement using degassed water should not deviate from the reference value ($\rho_{25} = 0.997043$ g/cm$^3$) by more than its specified error. Precision is a function of the repeatability and stability of the oscillator.
frequency. Density meters are able to achieve measurements with an error on the order of $1 \times 10^{-3}$ g/cm$^3$ to $1 \times 10^{-5}$ g/cm$^3$ and a repeatability of $1 \times 10^{-4}$ g/cm$^3$ to $1 \times 10^{-6}$ g/cm$^3$. For example, an instrument specified to $\pm 1 \times 10^{-4}$ g/cm$^3$ must display $0.9970 \pm 0.0001$ g/cm$^3$ in order to be suitable for further measurement, otherwise a readjustment is necessary. Calibration with certified reference materials should be carried out regularly.

**Procedure**

▲ Verify the viscosity of the sample to be analyzed. If the viscosity is $>$10 mPa·s and the oscillating transducer density meter is unable to provide a density correction based on the viscosity, use Method I (USP 1-Aug-2020).

Using the manufacturer’s instructions, perform the measurements using the same procedure as for Calibration. If necessary, equilibrate the liquid▲ (USP 1-Aug-2020) at $25^\circ$ before introduction into the tube to avoid the formation of bubbles and to reduce the time required for measurement. Factors affecting accuracy include the following:

- Temperature uniformity throughout the tube
- Nonlinearity over a range of density
- Parasitic resonant effects
- Viscosity, if the oscillating transducer density meters used do not provide automatic compensation of sample viscosity influence

▲ The oscillating transducer density meter provides the reportable value of the sample density at the selected temperature. The specific gravity of the sample is calculated from this measured density according to the following equation:

$$
\frac{\rho_{s,t}}{\rho_{w,t}} \cdot d_t' = \frac{\rho_{s,t}}{\rho_{w,t}}
$$

$\rho_{s,t} =$ density of the sample at $t'$

$\rho_{w,t} =$ density of water at $t$

[Note—The density of water used in the calculation of the specific gravity should be the accepted value of the density of water at $t$ (e.g., at $25^\circ$ use 0.9970 g/cm$^3$) rather than the value reported by the instrument.▲ (USP 1-Aug-2020)]

〈852〉 ATOMIC ABSORPTION SPECTROSCOPY

**INTRODUCTION**

Atomic absorption (AA) spectroscopy is an analytical method that supports qualification and/or quantification of elements. In this use, the AA method supports procedures that measure the absorbance of radiation at a characteristic wavelength by a vapor composed of ground state atoms. A typical instrument consists of a primary energy source that produces the spectrum of the element under examination, a monochromator, and a suitable detector.

For discussion of the theory and principles of measurements, see Atomic Absorption Spectroscopy—Theory and Practice (1852), a resource that may be helpful but is not mandatory.

**QUALIFICATION OF ATOMIC ABSORPTION SPECTROPHOTOMETERS**

Qualification of an AA spectrophotometer can be divided into three elements: installation qualification (IQ), operational qualification (OQ), and performance qualification (PQ); see also the general information chapter Analytical Instrument Qualification (1058).

**Installation Qualification**

The IQ requirements provide evidence that the hardware and software are properly installed in the desired location.

**Operational Qualification**

In OQ, an instrument’s performance is characterized using standards of known spectral properties to verify that the system operates within target specifications (see Table 1 and Table 2). The purpose of OQ is to demonstrate that instrument performance is suitable. OQ is a check of the key operational parameters performed following installation and following repairs and/or maintenance. The OQ tests in the following sections are typical examples only. Other tests and samples can be used to establish specifications for OQ. Instrument vendors often have samples and test parameters available as part of the IQ/OQ package.
Table 1. OQ Test and Acceptance Criteria for Flame AAS

<table>
<thead>
<tr>
<th>Test</th>
<th>Procedure</th>
<th>Acceptance Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>Aspirate a 0.3-µg/mL Zn standard and record the absorbance.</td>
<td>±20% of AU (^a) specified by instrument manufacturer</td>
</tr>
<tr>
<td>Linearity</td>
<td>Aspirate blank, 0.05-, 0.075-, 0.10-, 0.25-, and 0.50-µg/mL Zn standards. Generate calibration curve and record correlation coefficient (R).</td>
<td>Correlation coefficient NLT 0.995</td>
</tr>
<tr>
<td>Precision</td>
<td>Assay 5 separate replicates of the 0.10-µg/mL Zn standard versus the standard curve generated for the Linearity test. Calculate the %RSD of the 5 results in µg/mL.</td>
<td>%RSD (^b) NMT 3%</td>
</tr>
</tbody>
</table>

\(^a\) AU = absorption unit.
\(^b\) %RSD = % relative standard deviation.

Table 2. OQ Test and Acceptance Criteria for Graphite Furnace AAS

<table>
<thead>
<tr>
<th>Test</th>
<th>Procedure</th>
<th>Acceptance Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>Prepare a Cu standard and measure the characteristic mass as described by the manufacturer.</td>
<td>±20% of Cu characteristic mass as specified by the manufacturer</td>
</tr>
<tr>
<td>Linearity</td>
<td>Generate a calibration curve from a blank, 25-, 50-, 75-, and 100-µg/L Cu standards. Inject each standard in triplicate and record the %RSD.</td>
<td>Correlation coefficient NLT 0.995</td>
</tr>
<tr>
<td>Precision</td>
<td>Assay 5 separate replicates of the 50-µg/L Cu standard versus the standard curve generated for the Linearity test. Inject each replicate in triplicate.</td>
<td>%RSD of 5 replicates NMT 3%</td>
</tr>
</tbody>
</table>

\(^b\) %RSD of triplicate injections of each Cu standard NMT 5% (not including the blank)

Performance Qualification

PQ determines that the instrument is capable of meeting the user’s requirements for all the parameters that may affect the quality of the measurement.

Depending on typical use, the specifications for PQ may be different from the manufacturer’s specifications. For validated methods, specific PQ tests, also known as system suitability tests, can be used in lieu of PQ requirements.

Specific procedures, acceptance criteria, and time intervals for characterizing AA spectrophotometer performance depend on the instrument and intended application. Demonstrating stable instrument performance over extended periods of time provides some assurance that reliable measurements can be taken from test sample spectra using validated AA procedures.

PROCEDURE

Evaluate and select the type of material of construction, pretreatment, and cleaning of analytical labware used in AA analyses. The material must be inert and, depending on the specific application, resistant to caustics, acids, and/or organic solvents. For some analyses, diligence must be exercised to prevent the adsorption of analytes onto the surface of a vessel, particularly in ultra-trace analyses. Contamination of the sample solutions from metal and ions present in the container also can lead to inaccurate results.

For the analysis of a ubiquitous element, it is often necessary to use the purist grade of reagent or solvent available. Check all solutions (diluents, matrix modifier solutions, ionization suppression solutions, reactants, and others) for elemental contamination before they are used in an analysis.

Standard Solution

Prepare as directed in the individual monograph. [Note—Commercially available single- or multi-element standard solutions, traceable to the National Institute of Standards and Technology or to an equivalent national metrology organization, can be used in the preparation of standard solutions.] Standard solutions, especially those used for ultra-trace analyses, may have limited shelf life. Standard solutions should be retained for NMT 24 h unless stability is demonstrated experimentally.

The method of standard additions also can be used. This method involves adding a known concentration of the analyte element to the sample at no fewer than two concentration levels against an unspiked sample preparation. The instrument response is plotted against the concentration of the added analyte element, and a linear regression line is drawn through the data points. The absolute value of the x-intercept multiplied by any dilution factor is the concentration of the analyte in the sample.

Sample Solution

Prepare as directed in the individual monograph.

A variety of digestion techniques may be indicated to dissolve the sample. These may include hot-plate and microwave-assisted digestions, including open-vessel and closed-vessel approaches. Note that open-vessel digestion generally is not recommended for the analysis of volatile metals, e.g., selenium and mercury.
Analysis

Follow the procedure as directed in the individual monograph for the instrumental parameters. The instrument must be standardized for quantification at the time of use. The absorbance of standard solutions that bracket the target concentration is determined against an appropriate blank. The detector response is plotted as a function of the analyte concentration. When an analysis is performed at or near the detection limit, the analyst cannot always use a bracketing standard. This is acceptable for qualitative but not quantitative tests. Regression analysis of the standard plot should be used to evaluate the linearity of detector response, and individual monographs may set criteria for the residual error of the regression line.

To demonstrate the stability of the system’s initial standardization, the analyst must reassay a solution used in the initial standard curve as a check standard at appropriate intervals throughout the analysis of the sample set. Unless otherwise indicated in the individual monograph, the reassayed standard should agree with its expected value to within ±3% for an assay or ±20% for an impurity analysis.

Sample concentrations are calculated versus the working curve generated by plotting the detector response versus the concentration of the analyte in the standard solutions.

VALIDATION AND VERIFICATION

Validation

Validation is required when an AA method is intended for use as an alternative to the official procedure for testing an official article.

The objective of an AA procedure validation is to demonstrate that the measurement is suitable for its intended purpose, including quantitative determination of the main component in a drug substance or a drug product (Category I assays), quantitative determination of impurities or limit tests (Category II), and identification tests (Category IV). [Note—For definition of different categories, see Validation of Compendial Procedures (1225).] Depending on the category of the test, analytical procedure validation for AA may require the testing of linearity, range, accuracy, specificity, precision, detection limit, quantitation limit, and robustness. These analytical performance characteristics apply to externally standardized methods and to the method of standard additions.

General information chapter (1225) provides definitions and general guidance on analytical procedures validation without indicating specific validation criteria for each characteristic. The intention of the following sections is to provide the user with specific validation criteria that represent the minimum expectations for this technology. For each particular application, tighter criteria may be needed to demonstrate suitability for the intended use.

ACCURACY

For Category I assays or Category II tests, accuracy can be determined by conducting recovery studies with the appropriate matrix spiked with known concentrations of elements. It is also an acceptable practice to compare assay results obtained using the AA procedure under validation to those of an established analytical procedure. In standard addition methods, accuracy assessments are based on the final intercept concentration, not the recovery calculated from the individual standard additions. Validation criteria: 95.0%–105.0% mean recovery for the drug substance assay and the drug product assay, and 70.0%–150.0% mean recovery for the impurity analysis. These criteria apply throughout the intended range.

Precision

REPEATABILITY

The analytical procedure should be assessed by measuring the concentrations of six independently prepared sample solutions at 100% of the assay test concentration. Alternatively, three replicates of three separate sample solutions at different concentrations can be used. The three concentrations should be close enough that the repeatability is constant across the concentration range. If this is done, the repeatability at the three concentrations is pooled for comparison to the acceptance criteria. If validating a procedure by the method of standard additions, the precision criterion applies to the final experimental result, not the accuracy of the individual standard addition levels. Validation criteria: The relative standard deviation is NMT 5.0% for the drug substance assay, NMT 5.0% for the drug product assay, and NMT 20% for the impurity analysis.

INTERMEDIATE PRECISION

The effect of random events on the analytical precision of the procedure should be established. Typical variables include performing the analysis on different days, using different instrumentation, or having the method performed by two or more analysts. As a minimum, the analytical procedure should be assessed by performing the repeatability test in any combination of at least two of the conditions previously mentioned (totaling 12 measurements). Validation criteria: The relative standard deviation is NMT 8.0% for the drug substance assay, NMT 8.0% for the drug product assay, and NMT 25.0% for the impurity analysis.
SPECIFICITY

The procedure must be able to unequivocally assess each analyte element in the presence of components that may be expected to be present, including any matrix components.

Validation criteria: Demonstrated by meeting the accuracy requirement.

QUANTITATION LIMIT

The limit of quantitation (QL) can be estimated by calculating the standard deviation of NLT six replicate measurements of a blank solution, divided by the slope of a standard curve, and multiplying by 10. If validating a procedure using the method of standard additions, the slope of standards applied to a solution of the test material is used. Other suitable approaches can be used (see Section 1225).

A measurement of a test solution prepared from a representative sample matrix spiked at the estimated QL concentration must be performed to confirm accuracy. If validating a procedure using the method of standard additions, the validation criterion applies to the final experimental result, not the spike recovery of the individual standard addition levels.

Validation criteria: The analytical procedure should be capable of determining the analyte precisely and accurately at a level equivalent to 50% of the specification.

LINEARITY

A response curve between the analyte concentration and absorbance is prepared from NLT five standard solutions at concentrations encompassing the anticipated concentration of the test solution. The standard curve is then evaluated using appropriate statistical methods, such as a least-squares regression.

For experiments that do not yield a linear relationship between analyte concentration and AA response, appropriate statistical methods must be applied to describe the analytical response.

Validation criteria: Correlation coefficient ($R$), NLT 0.995 for Category I assays and NLT 0.99 for Category II quantitative tests.

RANGE

Range is the interval between the upper and lower concentrations (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy, and linearity. Range is demonstrated by meeting the linearity, precision, and accuracy requirements.

Validation criteria: For Category I tests, the validation range for 100.0% centered acceptance criteria is 80.0%–120.0%. For noncentered acceptance criteria, the validation range is 10.0% below the lower limit to 10.0% above the upper limit. For content uniformity, the validation range is 70.0%–130.0%. For Category II tests, the validation range covers 50.0%–120.0% of the acceptance criteria.

ROBUSTNESS

The reliability of an analytical measurement is demonstrated by deliberate changes to experimental parameters. For AA this can include but is not limited to sample preparation steps and heating programs, including atomization hold time or atomization temperature. Exercise caution when changing fuel and oxidant gas flows and burner hardware, because this could potentially create a flash-back condition.

Verification

U.S. Current Good Manufacturing Practices regulations [21 CFR 211.194(a)(2)] indicate that users of the analytical procedures, as described in USP–NF, are not required to validate these procedures if provided in a monograph. Instead, they must simply verify their suitability under actual conditions of use.

The objective of an AA procedure verification is to demonstrate that the procedure, as prescribed in a specific monograph, can be executed by the user with suitable accuracy, specificity, linearity, and precision using the instruments, analysts, and sample matrices available. According to Verification of Compendial Procedures (1226), if the verification of the compendial procedure by following the monograph is not successful, the procedure may not be suitable for use with the article under test. It may be necessary to develop and validate an alternative procedure as allowed in General Notices, 6.30 Alternative and Harmonized Methods and Procedures.

Verification of compendial AA methods should, at a minimum, include the execution of the validation parameters for specificity, linearity, accuracy, precision, and limit of quantitation, when appropriate, as indicated in Validation.

〈853〉 FLUORESCENCE SPECTROSCOPY

INTRODUCTION

Fluorescence is a two-step process that requires absorption of light at a specific wavelength (excitation) followed by emission of light, usually at a higher wavelength. The emission of light is termed fluorescence.
The most common type of fluorescent sample is a submicromolar transparent solution that absorbs light following the Beer–Lambert–Bouguer Law and fluoresces with an intensity that is directly proportional to the concentration, the absorptivity, and the fluorescence quantum yield of the fluorescent species or fluorophore.

Unlike absorption spectroscopy, where deviation from linearity is the exception, fluorescence linearity can be affected by a number of sample-related effects. For additional information, see Fluorescence Spectroscopy—Theory and Practice (1853).

Fluorescence methods also are termed background-free because little excitation light reaches the detector. This characteristic makes fluorescence detection highly sensitive, down to single-molecule detection in some cases. Fluorescence detection also can be highly specific because a fluorophore emits a characteristic emission pattern. Specificity and sensitivity are two of the more important strengths of fluorescence methods.

QUALIFICATION OF FLUORESCENCE INSTRUMENTS

Analysts ensure the suitability of a specific instrument for a given procedure by using a stepwise evaluation for the desired application from selection to instrument retirement: design qualification (DQ); installation qualification (IQ); an initial performance-to-specification qualification, also known as operational qualification (OQ); and an ongoing performance qualification (PQ). For additional information, see general chapter Analytical Instrument Qualification (1058).

DQ and IQ are not further considered in this chapter. The purpose of this section is to provide test methods and acceptance criteria to ensure that the instrument is suitable for its intended use (OQ) and that it will continue to function properly over extended time periods (PQ).

As with any spectrometric device, analysts must qualify a spectrofluorometer for both wavelength (x-axis) and relative intensity (y-axis or signal axis) accuracy and precision. They also must establish sensitivity. OQ should span the operational ranges required within the laboratory for both intensity and wavelength scales.

Instrument Operational Qualification

The tolerances given in both the instrument OQ and PQ are applicable for general use. Specifications for particular instruments and applications can vary depending on the analytical procedure used and the desired accuracy of the final result. Instrument vendors often have samples and test parameters available as part of the IQ/OQ package.

Wherever possible, analysts should use certified reference materials for purposes of calibration in the steps detailed below in preference to laboratory-prepared solutions. When certified reference materials are obtained from a recognized accredited source, they have independently verified traceable value assignments with associated calculated uncertainties.

Two general types of instrumental measurements are differentiated here: spectral (i.e., those that measure intensity versus wavelength) and fixed (i.e., those that measure intensity at a fixed wavelength and bandwidth).

CONTROL OF WAVELENGTHS

The level of confidence of measured peak positions is defined by wavelength accuracy for spectral measurements. Determination of the accuracy of many wavelengths across the desired wavelength range demonstrates if further calibration beyond a single point is needed. Multipoint calibration involves measuring wavelength biases at multiple wavelengths and correcting for the wavelength dependence of the bias. A single-point calibration often can be applied to the wavelength axis in an instrument’s software before data are collected, but a multipoint calibration may require that the correction be applied to spectra after they are collected.

For fixed measurements, the wavelength position and bandwidth must be reproducible. For filter-based wavelength selection, this requires that only the same filter be used when analysts compare data over time. If a different filter must be used (e.g., when data are compared across instruments and laboratories), then the transmission curves of the filters must be compared.

Wavelength precision should be determined over the operational range using at least six replicate measurements. The standard deviation should not exceed ±1 nm.

ATOMIC LINE SPECTRA

This procedure is described as the primary application because the emission lines produced from a discharge lamp are characteristic of the source element, and, as a fundamental physical standard, these wavelengths have been measured with an uncertainty of NMT ± 0.01 nm. In solution spectrofluorometry the wavelength bias required rarely exceeds 1.0 nm. For these reasons, the atomic line standard values are cited without uncertainty. The lamp should be placed at the source position in the spectrofluorometer.

A commonly employed low-pressure mercury lamp has a number of intense lines that cover a large part of the UV and visible range. Manufacturers often use two Xenon lines from the source at 260.5 and 541.9 nm as an internal calibration check, because the accuracy of both the excitation and emission monochromators can be verified and can be used for diagnostic purposes (see Table 1).

<table>
<thead>
<tr>
<th>Table 1. Elemental Line Spectra Wavelengths</th>
</tr>
</thead>
<tbody>
<tr>
<td>Element</td>
</tr>
<tr>
<td>---------</td>
</tr>
<tr>
<td>Hg</td>
</tr>
</tbody>
</table>

1 The rounded values are taken from ASTM Standard E388-04 (2009).
Table 1. Elemental Line Spectra Wavelengths (continued)

<table>
<thead>
<tr>
<th>Element</th>
<th>Wavelength (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xe</td>
<td>260.5</td>
</tr>
<tr>
<td>Hg</td>
<td>296.7</td>
</tr>
<tr>
<td>Hg</td>
<td>365.0</td>
</tr>
<tr>
<td>Hg</td>
<td>404.7</td>
</tr>
<tr>
<td>Hg</td>
<td>435.8</td>
</tr>
<tr>
<td>Xe</td>
<td>541.9</td>
</tr>
<tr>
<td>Hg</td>
<td>546.1</td>
</tr>
<tr>
<td>Hg</td>
<td>577.0</td>
</tr>
<tr>
<td>Hg</td>
<td>579.1</td>
</tr>
</tbody>
</table>

USE OF RARE EARTH OXIDE SOLUTIONS

This procedure uses a solution of a rare earth oxide prepared by dissolution in acid media. The most frequently used is holmium oxide in perchloric acid in combination with a diffuse reflector located at the sample position. Suitable certified reference materials are available commercially. The wavelength selector not being scanned should be removed; if removal is not practicable, it should be set to zero order (in this position a grating behaves like a mirror reflecting all wavelengths). The diffuse reflector is scanned with and without the rare earth sample in place, and the ratio of the two intensities is calculated to obtain an effective transmittance spectrum. Minima in the intensity ratio correspond to absorption peaks of the sample. For a 4% (w/w) solution of holmium oxide in perchloric acid at 1.0 nm spectral bandwidth and a path length of 1 cm, these minima are in Table 2.

<table>
<thead>
<tr>
<th>Wavelength (nm)</th>
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</thead>
<tbody>
<tr>
<td>241.1</td>
</tr>
<tr>
<td>249.9</td>
</tr>
<tr>
<td>278.1</td>
</tr>
<tr>
<td>287.2</td>
</tr>
<tr>
<td>333.5</td>
</tr>
<tr>
<td>345.4</td>
</tr>
<tr>
<td>361.3</td>
</tr>
<tr>
<td>385.6</td>
</tr>
<tr>
<td>416.3</td>
</tr>
<tr>
<td>451.4</td>
</tr>
<tr>
<td>467.8</td>
</tr>
<tr>
<td>485.2</td>
</tr>
<tr>
<td>536.6</td>
</tr>
<tr>
<td>640.5</td>
</tr>
</tbody>
</table>

If the operational range of the spectrophotometer lies outside 240–650 nm, other certified rare earth oxides or other solutions are used.

Didymium (a mixture of neodymium and praseodymium) is available as a traceable standard in both solution and glass presentations. Didymium is similar in preparation to the holmium materials and has useful peak characteristics in the 730–870 nm region. Useful peaks are found in the didymium solution at approximately 731.6, 740.0, 794.1, 799.0, and 864.4 nm.

USE OF POLYMETHYL METHACRYLATE-DOPED REFERENCES

This procedure uses solid reference materials manufactured by polymerization of a variety of fluorescent active aromatic ring compounds into an inert polymethylmethacrylate (PMMA) matrix. These materials are supplied as polished blocks for use in a standard cuvette holder (see Table 3).

2 NIST SRM 2034 is no longer available.
3 The rounded values are taken from the intrinsic wavelength standard absorption band data from Travis et al. J Phys Chem Ref Data 2005;34(1):41. The maximum 95% measurement uncertainty is ±0.06 nm.
### Performance Verification

Results from day-to-day testing of photostable intensity standards are used to verify the performance of an instrument. If the measured intensity does not change from that observed when the instrument was qualified, then instrument performance has not changed and remains qualified. Using such standards to determine an artifact-based or quasi-absolute intensity scale potentially enables measured intensities and instrument sensitivity to be compared over time or between instruments. Intensity measurements should be within the linear range of the instrument’s detection system before analysts attempt intensity comparisons, and will be affected by any instrumental effect directly related to the fluorescence signal, e.g., changes in source intensity, detector response, etc.

For instruments with filter-based wavelength selection, analysts use fluorescence standards for spectral correction to determine expected intensity differences caused by filters with different transmission profiles. By compensating for these intensity differences due to spectral mismatch, the analyst can determine a quasi-absolute intensity scale for these instruments. Analysts should approach instrument-to-instrument comparisons with particular caution because of the relatively large and difficult-to-quantify uncertainties involved.

#### USE OF LOW-CONDUCTIVITY (18-MΩ) WATER

The Raman band of water is used to measure signal-to-noise ratios in fluorescent instruments. The Raman band of water is inherently reproducible and does not degrade with time. Water is convenient to obtain in a pure state and allows interlaboratory comparisons to be made with a high level of confidence. No preparation or dilution is required. The Raman band is a low-level signal that provides a good test for both the optics and the electronics of an instrumental system.

The Raman band of water is not caused by fluorescence but is a result of Raman scattering. For water, the Raman band is always red-shifted $3382 \text{ cm}^{-1}$ relative to the excitation. This band usually is measured by excitation at 350 nm, resulting in a Raman peak at 397 nm, but radiation up to 500 nm also can be used as the excitation wavelength, and the corresponding emission peak is 602 nm.

#### USE OF INTENSITY STANDARDS

Several solid-doped fluorescent materials are available. These polymers or glasses enable the relative spectral correction and day-to-day performance qualification of fluorescent instruments across the UV, visible, and NIR regions from 320 to 830 nm. The high photostability of the materials makes them particularly useful as day-to-day intensity standards, even when spectral correction is not needed or when the excitation wavelength differs from that used for certification. A certified, steady-state emission spectrum is supplied with each certified reference material, along with the estimated total uncertainties. The reference is available in the form of a solid glass, standard-sized cuvette (12.5 mm × 12.5 mm × 45 mm) with three polished long faces for 90° detection and one frosted long face for front-face or epifluorescence detection.

Alternatively, analysts can use fluorophores in solutions that have been shown to be stable.

#### QUALITATIVE AND QUANTITATIVE FLUORESCENCE MEASUREMENTS

Two general classes of procedural measurements commonly are performed by fluorescence spectrometry: qualitative and quantitative.

**Qualitative Fluorescence Measurements**

Qualitative fluorescence measurements are used to detect the presence of particular analytes and yield a positive or negative answer. The excitation and emission wavelengths often are selected at the peak maximum of the fluorophore to be detected. The minimum amount of analyte needed for a positive result should be considered by the analyst to ensure that the method is appropriate for the particular application. The observation of fluorescence at the peak position above the limit of detection (usually 3 times the noise level) indicates a positive result.

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4 The red-shift value is taken from Parker CA. Raman spectra in spectrofluorimetry. *Analyst.* 1959;84:446–453.
5 Available from commercial vendors and from NIST as SRMs 2940 (orange emission), 2941 (green emission), 2942 (UV emission), 2943 (blue emission), and 2944 (red/NIR emission).
6 Commercial vendors provide a 1-mg/L solution of quinine sulfate dehydrate in 0.105 M perchloric acid that has been fully characterized by NIST as SRM 936a.
7 A series of day-to-day Intensity Standards is available from the German Federal Institute for Materials Research and Testing (BAM).
Quantitative Fluorescence Measurements

Quantitative fluorescence measurements are used to determine amounts or concentrations of analytes in unknown samples. These quantities may be determined in absolute units, such as moles or moles per L, or in relative units, such as the ratio of the concentrations of two fluorescent analytes contained in a single unknown solution. These determinations use the following proportionality relating fluorescence signal ($S$) at a given pair of excitation and emission wavelengths ($\lambda_{exc}, \lambda_{em}$) to fluorescent analyte concentration ($c$):

$$S \propto \frac{l_0}{\alpha} \times \Omega \times R_\Omega \times \Phi \times c$$

- $l_0$ = intensity of the excitation beam
- $\Omega$ = fraction of the fluorescence collected by the detection system
- $R_\Omega$ = responsivity of the detection system
- $\alpha$ = absorption coefficient
- $\Phi$ = fluorescence quantum yield
- $c$ = concentration of the fluorescent analyte

This linear proportionality with concentration applies to optically dilute samples (e.g., solutions with an absorbance of less than 0.05 at a path length of 1 cm).

GOOD SPECTROSCOPIC PRACTICE

Comparisons of a test specimen with a Reference Standard are best made at a peak of spectral emission for the compound of interest. Assays based on spectrophotometry give the commonly accepted wavelengths for excitation and peak spectral emission of the substance in question. Different spectrophotometers may show minor variation in the apparent wavelength of this peak. Comparisons should be made at the wavelength at which peak emission occurs. If this differs from the wavelength specified in the monograph by more than ±1 nm in the range of 200–400 nm or by more than ±2 nm in the range of 400–800 nm, recalibration of the instrument may be indicated.

Use of Reference Standards

With few exceptions, pharmacopeial spectrophotometric procedures provide results by comparison against a USP Reference Standard. This ensures measurement under identical conditions for the test specimen and the Reference Standard. These conditions could include wavelength setting, spectral bandwidth selection, cell placement and correction, and intensity levels. Analysts should establish and use appropriate cell corrections where required.

The terms *similar preparation* and *similar solution* in tests and assays that involve spectrofluorometry indicate that the Reference Standard should be prepared and observed in a manner that is identical to that used for the sample under test. Usually when a solution of the specified Reference Standard is prepared at (i.e., within 10% of) the desired concentration, the fluorescence intensity is calculated on the basis of the exact amount weighed out. If analysts have not used a previously dried specimen of the Reference Standard, they should correct this intensity on the anhydrous basis.

The expressions *concomitantly determine* and *concomitantly measured* as used in procedures that involve spectrophotometry indicate that the fluorescence of both the sample solution and the standard solution, relative to the specified test blank, are to be measured in immediate succession.

Sample Solution Preparation

For determinations using UV or visible spectrophotometry, the specimen generally is dissolved in a solvent. Unless otherwise directed in the monograph, determinations are made at room temperature by using a path length of 1 cm. Many solvents are suitable for these ranges, including water, alcohols, chloroform, lower hydrocarbons, ethers, and dilute solutions of strong acids and alkalis. Solvents should be free from contaminants that fluoresce in the spectral region under examination. For the solvent, water-free methanol or alcohol or alcohol that has been denatured by the addition of methanol but does not contain benzene or other interfering impurities should be used. Spectrophotometric-quality solvents that are guaranteed to be free from contaminants are available commercially from several sources, but some analytical reagent-grade organic solvents may contain traces of impurities that fluoresce strongly in the UV region. New lots of these solvents should be checked for their transparency, and analysts should take care to use the same lot of solvent for the preparation of the sample solution, the standard solution, and the blank. Solvents that do not have an interfering fluorescence signature at the wavelength(s) of interest should be used. In normal usage, the fluorescence baseline intensity should not be more than 2% of the expected measurement signal unless a larger value previously has been justified.

Assays in the visible region usually call for comparing concomitantly the fluorescence intensities produced by the sample solution with that produced by a standard solution that contains approximately an equal quantity of a USP Reference Standard. In some situations, it may be permissible to omit the use of a Reference Standard. This is true when spectrophotometric assays are made with routine frequency, when a suitable standard curve is available and is prepared with the appropriate USP Reference Standard, and when the substance assayed conforms to Beer–Lambert–Bouguer Law within the range of about 75%–125% of the final concentration used in the assay. Under these circumstances, the fluorescence intensity found in the assay can be interpolated on the standard curve, and the assay result can be calculated. Such standard curves should be confirmed frequently and always when a new spectrophotometer or new lots of reagents are put into use.

Published on March 26, 2020
VALIDATION AND VERIFICATION

Validation

Validation is required when a procedure based on fluorescence spectroscopy is intended for use as an alternative to the official procedure. The objective of validation is to demonstrate that the measurement is suitable for its intended purpose, including the following: quantitative determination of the main component in a drug substance or a drug product (Category I assays), quantitative determination of impurities or limit tests (Category II), and identification tests (Category IV). Depending on the category of the test (for additional information, see Table 2 in Validation of Compendial Procedures (1225)), the process for analytical procedure validation for fluorescence requires testing for linearity, range, accuracy, specificity, precision, detection limit, quantitation limit, and robustness. These analytical performance characteristics apply to externally standardized procedures and those that use standard additions. Chapter (1225) provides definitions and general guidance about analytical procedures validation without indicating specific validation criteria for each characteristic. The intention of the following sections is to provide the user with specific validation criteria that represent the minimum expectations for fluorescence technology. For each particular application tighter criteria may be needed in order to demonstrate suitability for the intended use.

ACCURACY

For Category I, Category II, and Category III procedures, accuracy is determined by conducting recovery studies with the appropriate matrix spiked with known concentrations of the analyte. Analysts also can compare assay results obtained using the fluorescence procedure under validation to those from an established analytical procedure. Validation criteria: 98.0%–102.0% mean recovery for a drug substance, 95.0%–105.0% mean recovery for a drug product assay, and 80.0%–120.0% mean recovery for impurity analysis. These criteria must be met throughout the intended range.

Precision

REPEATABILITY

Repeatability of the analytical procedure is assessed by measuring the concentrations of six independently prepared sample solutions at 100% of the assay test concentration. Alternatively, repeatability is assessed by measuring concentrations of three replicates of three separate sample solutions at different concentrations. The three concentrations should be sufficiently similar so that the repeatability is similar across the concentration range. If this is done, the repeatability at the three concentrations can be pooled for comparison to the acceptance criteria. Validation criteria: The relative standard deviation is NMT 1.0% for a drug substance, NMT 2.0% for a drug product assay, and NMT 20.0% for impurity analysis.

INTERMEDIATE PRECISION

The effect of random events on the analytical precision of the procedure should be evaluated. Typical variables include performing the analysis on different days, using different instrumentation, and having the method performed by two or more analysts. As a minimum, any combination of at least two of these factors totaling six experiments will provide an estimation of intermediate precision. Validation criteria: The relative standard deviation is NMT 1.0% for a drug substance, NMT 3.0% for a drug product assay, and NMT 25.0% for impurity analysis.

SPECIFICITY

In fluorescence measurements, specificity is ensured by the use of a Reference Standard wherever possible and is demonstrated by the lack of interference from other components present in the matrix. Validation criteria: Demonstrated by meeting the accuracy requirement

DETECTION LIMIT

Analysts can estimate the detection limit (DL) by calculating the standard deviation of NLT six replicate measurements of a blank solution and multiplying by 3.3. Alternatively, the standard deviation can be determined from the error of the intercept from a calibration curve or by demonstration that the signal-to-noise ratio is >3.3. Analysts must confirm the estimated DL by analyzing samples at the calculated concentration.

QUANTITATION LIMIT

Analysts can estimate the quantitation limit (QL) by calculating the standard deviation of NLT six replicate measurements of a blank solution and multiplying by 10. Alternatively, the standard deviation can be determined from the error at the intercept from a calibration curve or by demonstration that the signal-to-noise ratio is >10. A sample solution prepared from a representative sample matrix spiked at the required QL concentration is measured to confirm sufficient sensitivity and adequate precision. The observed signal-to-noise ratio at the required QL should be >10. Validation criteria: For the estimated limit of quantitation to be considered valid, the measured concentration must be accurate and precise at a level equal to or less than 50% of the specification.
LINEARITY

A response curve between the analyte concentration and the fluorescence signal is prepared from NLT five Standard solutions at concentrations that encompass the anticipated concentration of the sample solution. Analysts then should evaluate the standard curve for linearity using appropriate statistical methods such as a least-squares regression. Deviation from linearity can result from either instrumental or sample factors, or both, and can be reduced to acceptable levels by reduction of the analyte concentration and thereby the associated absorbance values.

Validation criteria: The correlation coefficient ($R$) must be NLT 0.995 for Category I assays and NLT 0.99 for Category II quantitative tests.

RANGE

The operational range of an analytical instrument (and the analytical procedure as a whole) is the interval between the upper and lower concentrations (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the instrumental response function has a suitable level of precision, accuracy, and linearity.

Validation criteria: For Category I tests, the validation range for 100.0% centered acceptance criteria is 80.0%–120.0%. For noncentered acceptance criteria, the validation range is 10.0% below the lower limit to 10.0% above the upper limit. For content uniformity, the validation range is 70.0%–130.0%. For Category II tests, the validation range covers 50.0%–120.0% of the acceptance criteria.

ROBUSTNESS

Analysts should demonstrate the reliability of an analytical measurement by deliberate changes to experimental parameters. For fluorescence these changes can include measuring the stability of the analyte under specified storage conditions, varying pH, removal of oxygen, and adding possible interfering species, to list a few examples. Analysts should determine robustness concurrently using a suitable design-of-experiments procedure.

Verification

Analytical procedures described in USP–NF do not require validation. Instead, a verification is used to determine a procedure’s suitability under actual conditions of use.

Thus the objective of fluorescence procedure verification is to demonstrate the suitability of a test procedure under actual conditions of use. Performance characteristics that verify the suitability of a fluorescence procedure are similar to those required for any analytical procedure. For additional information, see Verification of Compendial Procedures (1226) for a discussion of the applicable general principles. Verification should be performed using a reference material and a well-defined matrix. Verification of compendial fluorescence procedures should at a minimum include the execution of the validation parameters for specificity, accuracy, precision, and quantitation limit, when appropriate, as indicated in Validation.

Indirect Measurement Requirements

Some fluorescence procedures employ chromogenic reactions. Generally the requirements for the analytical performance characteristics should be used. In some instances the required accuracy and precision for the direct measurements may not be achievable. Under these circumstances, the accuracy and precision requirements may be widened by as much as 50%. However, any such widening must be justified on scientific grounds and with documented evidence. Under these circumstances, the amount of replication required to produce a scientifically sound reportable value may be increased.

Change to read:

(855) NEPHELOMETRY ▲▲ (USP 1-May-2019) TURBIDIMETRY

Add the following:

▲1. INTRODUCTION
2. TERMS AND DEFINITIONS
3. APPLICATIONS
4. INSTRUMENTATION
5. FORMAZIN TURBIDITY STANDARDS
   5.1 Preparation of the Formazin Standards
6. QUALIFICATION OF TURBIDIMETERS AND NEPHELOMETERS
   6.1 Calibration
   6.2 Stray Light
   6.3 Range of Measuring Capability
   6.4 Resolution
Nephelometry and turbidimetry are analytical techniques that are based on the principles of light-scattering phenomena. Light scattering is the physical phenomenon in which a beam of light changes its direction of propagation (known as deflection) as a result of interaction with sufficiently small matter particles. It has been established from the Maxwell electromagnetic theory that a prerequisite for scattering to occur is that the refractive indexes of the suspended particles must be different from those of the suspending liquid. The larger the difference, the more intense the scattering becomes. There are two types of light scattering: 1) elastic scattering, in which the wavelength of the scattered light and incident light are the same; and 2) inelastic light scattering, in which the wavelength of the scattered light and incident light are different. Only the first type of light scattering (elastic) is relevant to nephelometry and turbidimetry.

In turbidimetry, the intensity of the transmitted light is measured and the attenuation of the intensity of incident light as a result of scattering is measured at the direction of incident light (i.e., 0°) and compared to the intensity of incident light (blank measurement). The measured property is an indirect measurement of the scattering effect of the suspended particles and is referred to as turbidance. Any absorbance of light by the suspended sample will result in additional attenuation of light intensity (see Ultraviolet-Visible Spectroscopy and Ultraviolet-Visible Spectroscopy—Theory and Practice). Hence, it is important to ensure that the material being measured does not absorb light at the measurement wavelength. Indeed the equations governing absorption and turbidimetry are the same (albeit with different values for the attenuation constants). In nephelometric techniques, the intensity of the scattered light at a 90° angle from the propagation direction of the incident light is measured. Therefore, a nephelometric measurement is a direct measurement of the scattering effect of suspended matter.

### 2. TERMS AND DEFINITIONS

Terms commonly used in describing turbidimetric and nephelometric techniques are:

- **Turbidance** (symbol, \( S \)): A measure of the decrease of the transmitted incident light beam intensity as a result of the light-scattering effect of suspended particles. The amount of suspended matter may be measured by observation of either the transmitted light (turbidimetry) or the scattered light (nephelometry).

\[
\log \frac{l_0}{l_t} = kbc = T
\]

- **Turbidity** (symbol, \( \tau \)): In turbidimetric measurements, the turbidity is the measure of the decrease in incident beam intensity/unit length of a given suspension. The International Organization for Standardization defines turbidity as “the reduction of transparency of a liquid caused by the presence of undissolved matter”.

- **Turbidity Measurement Units**: The turbidity units are stated using a descriptor which indicates the method of measurement.

- **Nephelometric Turbidity Units (NTUs)**: When the turbidity is measured using a nephelometer, which measures the scattered light at a 90° angle from the direction of propagation of incident light, the units of turbidity are called nephelometric turbidity units (NTUs). The magnitude of NTU is defined based on the turbidity generated by primary formazin standard (a suspension made by mixing solutions of hydrazine sulfate and hexamethylenetetramine in water). Safer polymer-bead suspensions are now commercially available and are recognized as an acceptable alternative. However, all those standards are traced to formazin. The primary formazin standard solution has been assigned a turbidity of 4000 NTUs.

Other recognized units for turbidity include the formazin turbidity unit (FTU) and the formazin nephelometric unit (FNU). These units are equivalent to NTU for the range from 0–40 NTUs.
3. APPLICATIONS

Turbidimetric and nephelometric techniques have applications that include 1) concentration determination of solutions and/or suspensions (determination of several cations and anions by precipitating and suspending the resulting precipitate at well-controlled reaction parameters); 2) measurement of the degree of turbidity of turbid solutions or suspensions; 3) determination of weight-average molecular weights and dimensions of polydisperse systems in the molecular weight range from 1000 to several hundred million; 4) measurement of immunoassays' reaction kinetics or kinetics of immunoprecipitations (rate nephelometry); 5) monitoring of cell and bacteria growth; and 6) particle size distribution determination of suspended material, particle counting, etc.

Rate nephelometry is widely used for vaccine components assays and/or quantitation of components in blood serum. It is also used for host cell protein qualification in recombinant biopharmaceuticals. When using the technique, the measurement of the change in the light-scattering response by antigen–antiserum or antigen-purified antibody complexes is used to calculate the amount of antigen (Ag) or antibody (Ab) responsible for the immunological Ab-Ag precipitation reaction or agglutination reaction. Often the antigens under consideration are linked covalently or adsorbed to polymeric microspheres to increase the scattering efficiency; the resulting technique is known as “particle-enhanced immunoassay”. Although the technique is described as nephelometry, usually both scattered and transmitted light are measured using the ratio instruments.

Nephelometric measurements are more reliable in low turbidity ranges (relatively low concentration of the scattering medium). In this range, a linear relationship is observed between the sample concentration and the detector’s signal intensity expressed as NTU. As the concentration increases, so does the incidence of multiple scattering that deviates the response from the linearity. The maximum NTU value, which supports a reliable linearity relationship, is in the range of 1750–2000 NTUs. Turbidimetry is preferred for higher turbidity ranges (concentrations of the scattering media). To achieve consistent results, all measurement variables must be carefully controlled. Where such control is possible, extremely dilute suspensions may be measured.

Change to read:

4. INSTRUMENTATION

Instruments used for turbidimetric and nephelometric measurements are called turbidimeters and nephelometers, respectively. Generally, these instruments consist of a mercury lamp with filters for the strong green or blue lines, a shutter, a set of neutral filters with known transmittance, and a sensitive photomultiplier, which can be mounted fixed at 0° or at a 90° angle from the incident light propagation direction, or on an arm that can be rotated around the solution cell and set at any angle from −135° to 0° to +135° by a dial outside of the light-tight housing. Solution cells are of various shapes, such as square for measuring 90° scattering; semioctagonal for 45°, 90°, and 135° scattering; and cylindrical for scattering at all angles (see Figure 1).

Figure 1. Representative nephelometric (turbidimetric) instrument. Note that Detector 2 may be mounted on a movable arm.

Turbidity also can be measured with a standard photoelectric filter photometer or spectrophotometer, preferably with illumination in the blue portion of the spectrum. Nephelometric measurements require an instrument with a photocell placed so as to receive scattered, rather than transmitted, light. Because this is the same geometry used in fluorometers, they can be used as nephelometers by proper selection of filters. A ratio turbidimeter combines the technology of 90° nephelometry and turbidimetry. It contains photocells that receive and measure scattered light at a 90° angle from the sample as well as receiving and measuring the forward scatter in front of the sample. It also measures light transmitted directly through the sample. Linearity is attained by calculating the ratio of the 90° angle scattered light measurement to the sum of the forward scattered light measurement and the transmitted light measurement. The benefit of using a ratio turbidimetric system is that the measurement of stray light becomes negligible. In addition, the determination of turbidity of colored suspensions is done exclusively using turbidimetric or nephelometric instruments with ratio mode because this procedure compensates for the attenuation of light as the result of the suspension color. Typically, the light source in these instruments is a tungsten lamp with most of the light intensity at about 550 nm operating at the filament temperature of 2700 K. Other suitable light sources are also available. Typically, the detectors are silicon diodes and photomultipliers. An alternative for eliminating the color effect involves using an infrared light-emitting diode as a light source, which yields an emission maximum centered at about 860 nm.
and a spectral bandwidth of 60 nm. When laser light sources are also used, especially in nephelometric instruments, the technique is commonly known as "laser nephelometry". The advantage of using laser nephelometers is the significant improvement in signal-to-noise ratio at very low detection levels. Usually the light source is a laser diode with a working wavelength at 660 nm. The high-power density of the laser beam gives rise to higher scattered intensity from smaller particles. Combined with a light trap, which absorbs the unscattered light, the system lowers the stray light significantly. When the use of a nephelometer or turbidimeter is indicated for a procedure in a monograph, instruments working in ratio mode may be used instead.

Change to read:

## 5. Formazin Turbidity Standards

Formazin is the only known primary turbidity standard. All other standards are secondary and must be traced to formazin. The primary standard is defined in the *IUPAC Compendium of Chemical Terminology, 2nd ed. (the Gold Book)* as a suspension prepared by the user from traceable materials using well-defined methodologies and conditions.

Formazin suspension has many features that ensure its suitability as a primary standard. It can be consistently and accurately prepared from reagent-grade starting materials. The suspension consists of random polymers with different lengths and random configurations, which result in moieties of varying shapes and sizes ranging from less than 0.1 μm to more than 10 μm. Although the polymer chain length distribution has been shown to vary from preparation to preparation, the overall resulting turbidity has been very reproducible.

### 5.1 Preparation of the Formazin Standards

**[Note—All procedures described below must be performed at 20 ± 2°C (see Volumetric Apparatus 31).]**

- **Hydrazine sulfate solution:** Dissolve 1.000 g of ACS grade hydrazine sulfate (N₂H₄·H₂SO₄) in particle-free water in a 100-mL Class A volumetric flask and dilute with particle-free water to volume. Allow this solution to stand for 4–6 h.

- **Primary formazin standard:** Dissolve 2.50 g of analytical grade hexamethylenetetramine [(CH₂)₆N₄] in 25.0 mL of particle-free water in a 100-mL flask. Add 25.0 mL of hydrazine sulfate solution using a Class A 25-mL “to deliver” pipette and mix thoroughly. Allow the preparation to stand for 48 h at 25 ± 1°C before using. The resulting suspension is stable for 2 months.

- **Formazin stock standard suspension 1:** Using a 15-mL Class A “to deliver” pipette, transfer 15 mL of the Primary formazin standard to a 1-L volumetric flask, and dilute with particle-free water to volume and mix. The resulting suspension has a turbidity of 60 NTU.

- **Formazin stock standard suspension 2:** Using a 50-mL Class A “to deliver” pipette, transfer 50 mL of Primary formazin standard to a 200-mL volumetric flask, and dilute with particle-free water to volume and mix. The resulting suspension has a turbidity of 1000 NTUs.

- **Formazin reference suspensions:** Prepare by mixing in a 100-mL volumetric flask, portions of the respective Formazin stock standard suspension and particle-free water according to Table 1.

<table>
<thead>
<tr>
<th>Formazin Reference Suspensions</th>
<th>Formazin Stock Standard Suspension 1 Volume (mL)</th>
<th>Formazin Stock Standard Suspension 2 Volume (mL)</th>
<th>Particle-Free Water Volume (mL)</th>
<th>Turbidity Value (NTU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference suspension I</td>
<td>5.0</td>
<td></td>
<td>95.0</td>
<td>3</td>
</tr>
<tr>
<td>Reference suspension II</td>
<td>10.0</td>
<td></td>
<td>90.0</td>
<td>6</td>
</tr>
<tr>
<td>Reference suspension III</td>
<td>30.0</td>
<td></td>
<td>70.0</td>
<td>18</td>
</tr>
<tr>
<td>Reference suspension IV</td>
<td>50.0</td>
<td></td>
<td>50.0</td>
<td>30</td>
</tr>
<tr>
<td>Stock standard suspension I</td>
<td>100</td>
<td></td>
<td>0</td>
<td>60</td>
</tr>
<tr>
<td>Reference suspension VI</td>
<td></td>
<td>10.0</td>
<td>90.0</td>
<td>100</td>
</tr>
<tr>
<td>Reference suspension VII</td>
<td></td>
<td>20.0</td>
<td>80.0</td>
<td>200</td>
</tr>
<tr>
<td>Reference suspension VIII</td>
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<td>40.0</td>
<td>60.0</td>
<td>400</td>
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<tr>
<td>Reference suspension IX</td>
<td></td>
<td>70.0</td>
<td>30.0</td>
<td>700</td>
</tr>
<tr>
<td>Stock standard suspension II</td>
<td></td>
<td>100</td>
<td>0</td>
<td>1000</td>
</tr>
</tbody>
</table>

### 6. Qualification of Turbidimeters and Nephelometers

The suitability of a specific instrument for a given procedure is ensured by a stepwise life cycle evaluation for the desired application from selection to instrument retirement. The qualification comprises three components: 1) installation qualification (IQ), 2) operational qualification (OQ), and 3) performance qualification (PQ) (see *Analytical Instrument Qualification* 1058).
The purpose of this section is to provide test methods and acceptance criteria to ensure that the instrument is suitable for its intended use (OQ), and that it will continue to function properly over extended time periods (PQ). As with any spectrometric device, a turbidimetric and nephelometric spectrometer must be qualified for both wavelength (x-axis, if not fixed) and photometric (y-axis, or signal axis) accuracy and precision, and meet the requirements for the stray light. OQ is carried out across the operational ranges required within the laboratory for both the absorbance and wavelength scales.

Acceptance criteria for critical instrument parameters that establish “fitness for purpose” are verified during IQ and OQ. Specifications for particular instruments and applications can vary depending on the analytical procedure used and the desired accuracy of the final result. Instrument vendors often have samples and test parameters available as part of the IQ/OQ package.

Wherever possible in the procedures detailed as follows, primary reference standards or certified reference materials (CRMs) are to be used. Formazin is the only primary reference standard used in turbidimetry and nephelometry. All the other standards, including the CRMs, must be correlated to formazin. The CRMs should be obtained from a recognized accredited source and include independently verified traceable value assignments with associated calculated uncertainty. CRMs must be kept clean and free from dust. Recertification should be performed periodically to maintain the validity of the certification.

6.1 Calibration

All of the turbidimetric and nephelometric instruments are calibrated against standards of known turbidity. The instrument must be calibrated using formazin turbidity standards prior to its first time use and at least every 3 months or as specified by the vendor. Calibration is performed using at least four formazin turbidity standards whose turbidity proportionally covers the range of interest. Many instrument manufactures provide calibration verification standards. They usually consist of sealed sample cells filled with a latex suspension or with metal oxide particles in polymer gel. These standards must be used only for checking the calibration in the time intervals between the instrument recommended calibrations.

6.2 Stray Light

Stray light (stray radiant energy) is a very significant error source, especially for measurements in the range of the lower turbidity readings. It is defined as external light that reaches the detector without being scattered from the sample. There are several sources of stray light including the inherent cell surface imperfections, reflections from within the cell that are unaccounted for, optical system parts, light sources, and, to a smaller degree, the electronics fluctuations. Although there are many design features that instrument vendors use to minimize the stray light, a complete mitigation of the stray light cannot be achieved. Unlike spectrophotometric measurements, the stray light cannot be compensated for in turbidimetry. The stray light must be measured and the values should be within the specification range set by the vendor of the particular instrument or <0.15 NTUs for the measurement in the range of 0–10 NTUs and 0.5 NTUs for the measurements in the range of 10–1100 NTUs, whichever is smaller.

6.3 Range of Measuring Capability

The instrument must be able to measure the turbidity in the range of 0.01–1100 NTUs or from 50%–200% of the target turbidity. To demonstrate the linearity for the intended measurements range, choose at least four appropriate reference suspensions from Table 1.

6.4 Resolution

Instrument resolution must be 0.01 NTU or less for the measurements range of 0–9.99 NTUs; 0.1 NTU or less for the measurements range of 10–99.9 NTUs; and 1 NTU for the measurements above 100 NTUs.

6.5 Accuracy

The instrument reading accuracy must be ±10% of the reading + 0.01 NTU for the measurement range from 0–19.9 NTUs, and ±7.5% of the reading for the measurement range from 20–1100 NTUs.

6.6 Performance Qualification

The instrument PQ is accomplished periodically or as needed between the calibrations. Primary turbidity standards (formazin) or secondary calibration verification standards (latex suspensions or metal oxide particles in polymer gels contained in sealed sample cells) supplied by instrument manufacturers may be used.

7. PROCEDURES

7.1 Turbidimetric Procedures

SAMPLE CELL PREPARATION

The sample cells for sample measurements must be clean. Follow the sample cell or instrument manufacturer recommendations for cleaning the sample cells appropriately. For low turbidity measurements it is a good practice to use a
single-indexed sample cell or a flow cell, which help ensure adequate precision and repeatability of the measurements. Using particle-free water, find the sample cell orientation in the sample cell holder that gives the lowest reading. For higher values of turbidity, different sample cells may be used. However, the sample cells must be matched (the difference in readings for a standard prepared at nominal sample concentration from two different sample cells must be within ±0.005 NTU or below the measurement precision requirement, whichever is lower).

**SAMPLE PREPARATION**

Prepare the samples as prescribed in the individual monograph. Carefully mix the samples thoroughly by swirling or inverting the volumetric flask slowly several times. Avoid shaking or stirring since it may introduce bubbles. Degassing the samples helps to improve the measurements. For degassing, the samples could stand for several minutes or a vacuum could be applied, or they could be gently sonicated using an ultrasonic bath. After degassing, let the samples stand for several minutes and mix again by carefully inverting two to three times. Transfer the sample to the sample cell and take the readings.

**USE OF FLOW CELLS**

Flow cells are mainly used for low turbidity measurements for samples with small particles. When such cells are used, the sample is introduced by carefully pouring it down the interior edge of the inlet reservoir.

In practice, it is advisable to ensure that settling of the particles being measured is negligible. This is usually accomplished by including a protective colloid in the liquid-suspending medium. It is important that results be interpreted by a comparison of readings with those representing known concentrations of suspended matter, produced under precisely the same conditions.

**7.2 Nephelometric Procedures**

Nephelometric procedures are performed similarly to turbidimetric procedures for both direct measurements and measurements in the ratio mode as described above.

**RATE NEPHELOMETRIC PROCEDURES**

The overall procedure for monitoring the progress of the reaction consists of three well-defined steps: 1) record a baseline reading of the turbidity of the medium (blank); 2) record the turbidity after the first reagent (antigen) is added, which results in an increase of the turbidity until a plateau is reached; and 3) add the second reagent (antibody), which results in another turbidity increase and a second plateau followed by a final turbidity increase that continues until a third plateau is reached. The measurement zone is selected from the addition of the antibody until the third plateau, depending on the purpose of the assay and the respective component concentrations. **Kinetic nephelometry** and **endpoint nephelometry** are two general procedures that are used for quantifying the immune complexes formed in the immunoassay methods (also known as immunonephelometry because the measured turbidity is due to immunocomplexes that are formed). For each procedure, there are several parameters that need to be optimized in each individual application. The main parameters are 1) with or without particle enhancement; 2) particle types, sizes, and respective optimum wavelength, if applicable; 3) monitoring reaction kinetic or endpoint; 4) antibody/antigen under consideration and, related to that, the optimum level of antigen loading; 5) buffers and other ionic species and respective optimal pH; 6) type and concentration of polymers used to modify the solubility of proteins; and 7) temperature and other environmental factors. Generally these parameters are optimized during the method development and the values are given in specific monograph(s) and/or chapter(s) as applicable.

**Kinetic nephelometry:** The kinetic nephelometry is advantageous compared to the endpoint nephelometry mainly because of the capability to take a sample blank reading in addition to a reagent blank reading. This procedure assesses the rate of the immunocomplex formation based on the increased intensity response of the scattered light of the chosen wavelength. The reaction kinetic may be monitored continuously or a certain number of data points may be taken, depending on the time response of the instrument used and the type of application. At times it may involve only two data points; however, proper care must be exercised because the choice of point selection can influence the overall accuracy in cases where differences in reaction kinetics exist between samples and calibrating standards. Careful consideration should be given to the appropriate choice of specificity control strategy.

**Endpoint nephelometry:** In this method, an initial measurement is performed before adding the reagent, which represents the blank reading. A second measurement is performed after the immune complex is formed after approximately 60 min. The difference between these two measurements is proportional to the content of the component being assayed.

**8. VALIDATION AND VERIFICATION**

**8.1 Validation**

Validation is required when a nephelometric/turbidimetric method is intended for use as an alternative to the official procedure for testing an official article. The objective of nephelometric/turbidimetric method validation is to demonstrate that the measurement is suitable for its intended purpose, including quantitative determination of the main component in a drug substance or a drug product (Category I assays), quantitative determination of impurities or limit tests (Category II), and identification tests (Category IV). Depending on the category of the test (see Validation of Compendial Procedures (1225), Table 2), the analytical method validation process for nephelometry/turbidimetry requires testing for accuracy, precision, specificity,
detection limit (DL), quantitation limit (QL), linearity, range, and robustness. These analytical performance characteristics apply to externally standardized procedures and those that use standard additions.

Validation of Compendial Procedures (1225) provides definitions and general guidance on analytical procedures validation without indicating specific validation criteria for each characteristic. The intention of the following sections is to provide the user with specific validation criteria that represent the minimum expectations for this technology. For each particular application, tighter criteria may be needed in order to demonstrate suitability for the intended use.

**ACCURACY**

For Category I, II, and III procedures, accuracy can be determined by conducting recovery studies with the appropriate matrix spiked with known concentrations of the analyte. Analysts can also compare the assay results obtained using the nephelometric/turbidimetric procedure under validation to those from an established analytical procedure.

**Validation criteria:** 98.0%–102.0% mean recovery for the drug substances, 95.0%–105.0% mean recovery for the drug product assay, and 80.0%–120.0% mean recovery for the impurity analysis. These criteria are met throughout the specified range.

**PRECISION**

**Repeatability:** The repeatability of the analytical procedure is assessed by measuring the concentrations of six independently prepared sample solutions at 100% of the assay test concentration. Alternatively, it can be assessed by measuring the concentrations of three replicates of three separate sample solutions at different concentrations. The three concentrations should be close enough so that the repeatability is constant across the concentration range. If this is done, the repeatability at the three concentrations is pooled for comparison to the acceptance criteria.

**Validation criteria:** The relative standard deviation is NMT 1.0% for the drug substance, NMT 2.0% for the drug product assay, and NMT 20.0% for the impurity analysis.

**Intermediate precision:** The effect of random events on the analytical precision of the method must be established. Typical variables include performing the analysis on different days, using different instrumentation, and/or having the method performed by two or more analysts. At a minimum, any combination of at least two of these factors totaling six experiments will provide an estimation of intermediate precision.

**Validation criteria:** The relative standard deviation is NMT 1.5% for the drug substance, NMT 3.0% for the drug product assay, and NMT 25.0% for the impurity analysis.

**SPECIFICITY**

In nephelometric/turbidimetric measurements, specificity is demonstrated by the lack of interference from other components present in the matrix (other components of the matrix produce a true solution).

**DETECTION LIMIT**

The DL can be estimated by calculating the concentration of a solution that would give the signal-to-noise ratio of $\geq 3.3$. The estimated DL must be confirmed by analyzing samples at the calculated concentration.

**QUANTITATION LIMIT**

The QL can be estimated by calculating the concentration of a solution that would give the signal-to-noise ratio of $\geq 10.0$. The estimated QL must be confirmed by analyzing samples at the calculated concentration. Measurement of a test solution prepared from a representative sample matrix spiked at the required QL concentration must be performed to confirm sufficient sensitivity and adequate precision. The observed signal-to-noise ratio at the required QL should be $>10$.

**Validation criteria:** For the estimated limit of quantitation to be considered valid, the measured concentration must be accurate and precise at a level $\leq 50\%$ of the specification.

**LINEARITY**

A linear relationship between the analyte concentration and measured turbidity response must be demonstrated by preparation of at least four standard solutions at concentrations encompassing the anticipated concentration of the test solution. The standard curve is then evaluated using appropriate statistical methods such as a least-squares regression. Deviation from linearity results from instrumental or sample factors, or both, can be reduced to acceptable levels by reducing or increasing the analyte concentration, thereby respectively decreasing or increasing the turbidity readings to within the nephelometer/turbidimeter instrument linearity range.

**Validation criteria:** The correlation coefficient ($R$) must be NLT 0.995 for Category I assays and NLT 0.99 for Category II quantitative tests.

**RANGE**

The operational range of an analytical instrument (and the analytical procedure as a whole) is the interval between the upper and lower concentrations (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the instrumental response function has a suitable level of precision, accuracy, and linearity.
Validation criteria: For Category I tests, the validation range for 100.0% centered acceptance criteria is 80.0%–120.0%. For non-centered acceptance criteria, the validation range is 10.0% below the lower limit to 10.0% above the upper limit. For Category II tests, the validation range covers 50.0%–120.0% of the acceptance criteria.

**ROBUSTNESS**

The reliability of an analytical measurement is demonstrated by deliberate changes to experimental parameters. For nephelometry/turbidimetry this can include, for example, measuring the stability of the analyte under specified storage conditions, varying pH, and adding possible interfering species. Robustness is determined concurrently using a suitable design for the experimental procedure.

### 8.2 Verification

Current U.S. Good Manufacturing Practices regulations [21 CFR 211.194(a)(2)] indicate that users of analytical procedures described in the *U.S. Pharmacopeia* and *National Formulary* are not required to validate these procedures if provided in a monograph. Instead, they simply must verify their suitability under actual conditions of use. The objective of nephelometric/turbidimetric procedure verification is to demonstrate the suitability of a test procedure under actual conditions of use. Performance characteristics that verify the suitability of a nephelometric/turbidimetric procedure are similar to those required for any analytical procedure. A discussion of the applicable general principles is found in *Verification of Compendial Procedures* (1226). Verification is usually performed using a reference material and a well-defined matrix. Verification of compendial nephelometric/turbidimetric procedures includes, at minimum, the execution of the validation parameters for specificity, accuracy, precision, and QL, when appropriate, as indicated in 8.1 Validation.

### (857) ULTRAVIOLET-VISIBLE SPECTROSCOPY

**INTRODUCTION**

For the purposes of this chapter, an ultraviolet-visible (UV-Vis) spectrometer is defined as an optical system capable of producing monochromatic radiation in the range of 200–780 nm and as a device capable of detecting the optical transmittance, usually expressed in absorbance (A), whose primary function is to measure the stated absorbance/transmittance at defined wavelength(s). *(Note—A UV-Vis spectrometer may also be referred to as a spectrophotometer or absorption spectrometer.)*

This functionality may be extended to incorporate multiple channel measurements, either by instrumental design or with time, e.g., in dissolution or kinetic studies.

Chromatographic detectors are specifically excluded from this chapter.

For essential supporting information on the best practices and principles of measurements, see Ultraviolet-Visible Spectroscopy — Theory and Practice (1857).

The suitability of a specific instrument for a given procedure is ensured by a stepwise life cycle evaluation for the desired application from selection to instrument retirement: design qualification (DQ); installation qualification (IQ); an initial performance-to-specification qualification, also known as operational qualification (OQ); and an ongoing performance qualification (PQ). For more details, see Analytical Instrument Qualification (1058). Acceptance criteria for “fitness for purpose” are based on the uncertainty of the reference material and the performance specification of the instrument.

**Principles of Measurement**

UV-Vis spectra are derived when the interaction between incident radiation and the electron cloud in a chromophore results in an electronic transition involving the promotion of one or more of the outer shell or the bonding electrons from a ground state into a state of higher energy. The UV and visible spectral bands of substances generally are broad and do not possess a high degree of specificity for compound identification. Nevertheless, they are suitable for quantitative assays and, for many substances, are useful as an additional means of identification.

In the Beer–Lambert law the absorbance (A) of a solution at given wavelength, λ, is defined as the logarithm to base 10 of the reciprocal of the transmittance (T):

$$A = \log_{10} \left( \frac{1}{T} \right) \quad \text{and} \quad T = \frac{l}{l_0}$$

*T* = transmittance

*l* = intensity of the transmitted radiation at the same wavelength λ

*l₀* = intensity of the incident radiation at wavelength λ

In the absence of any other physical or chemical factors, A is proportional to path length, b, through which the radiation passes, and to the concentration, c, of the substance in the solution in accordance with the following:

$$A = \epsilon \cdot cb$$
\[ \varepsilon = \text{molar absorptivity} \]
\[ c = \text{solute concentration (mol/L)} \]
\[ b = \text{path length (cm)} \]

If the concentration, \( c \), is expressed in g/L, the constant \( \varepsilon \) becomes \( \alpha \), which is called the absorptivity.

The expression \( A_{\lambda}^{1\% 1\text{ cm}} \), which represents the specific absorbance of a dissolved substance, refers to the absorbance of a 10-g/L solution (1%, m/v) in a 1-cm cell measured at a defined wavelength so that:

\[ A_{\lambda}^{1\% 1\text{ cm}} = 10\alpha = 10\varepsilon / M \]

\[ \alpha = \text{absorptivity} \]
\[ \varepsilon = \text{molar absorptivity} \]
\[ M = \text{molar concentration of the solution} \]

When solutions are observed in 1-cm cells, concentrations of about 10 µg/mL often will produce absorbances of 0.2–0.8 in the UV or visible region.

Change to read:

**QUALIFICATION OF UV-VIS SPECTROMETERS**

The purpose of this section of the chapter is to provide test methodologies and acceptance criteria to ensure that the instrument is suitable for its intended use (OQ), and that it will continue to function properly over extended time periods as part of PQ. As with any spectrometric device, a UV-Vis spectrometer must be qualified for both wavelength (x-axis) and photometric (y-axis, or signal axis) accuracy and precision, and the fundamental parameters of stray light and resolution must be established. OQ is carried out across the operational ranges required within the laboratory for both the absorbance and wavelength scales.

**Installation Qualification**

Documented proof of the IQ requirements provide evidence that the hardware and software are properly installed in the desired location.

**Operational Qualification**

Acceptance criteria for critical instrument parameters that establish “fitness for purpose” are verified during IQ and OQ. Specifications for particular instruments and applications can vary, depending on the analytical procedure used and the desired accuracy of the final result. OQ should establish suitable control over the operational range of wavelength, absorbance, and the evaluation of stray light and resolution, i.e., spectral bandwidth (SBW). Instrument vendors often have reference materials and protocols available as part of the IQ/OQ package.

Wherever possible in the procedures detailed as follows, certified reference materials (CRMs) are to be used in preference to laboratory-prepared solutions. These CRMs should be obtained from a recognized accredited source and include independently verified, traceable value assignments with associated calculated uncertainty. CRMs must be kept clean and free from dust. Recertification should be performed periodically to maintain the validity of the certification.

**Control of Wavelengths**

Ensure that the accuracy of the wavelength axis (x-axis) over the intended operational range is correct within acceptable limits. The control of wavelength OQ must include at least one method in each operational range where it is intended for use. The options and acceptance criteria are summarized in Table 1.

**Table 1. Wavelength Ranges and Procedures for Control of Wavelengths**

<table>
<thead>
<tr>
<th>Method</th>
<th>UV (200–400 nm)</th>
<th>Vis (400–780 nm)</th>
<th>Vis/NIR (400–900 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mercury (Hg) emission lines</td>
<td>Accuracy ±1 nm Precision ≤0.5 nm</td>
<td>Accuracy ±2 nm Precision ≤0.5 nm</td>
<td>—</td>
</tr>
<tr>
<td>Deuterium (D₂) emission lines</td>
<td>—</td>
<td>Accuracy ±2 nm Precision ≤0.5 nm</td>
<td>—</td>
</tr>
<tr>
<td>Cerium oxide solutions</td>
<td>Accuracy ±1 nm Precision ≤0.5 nm</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Holmium oxide solutions or glasses</td>
<td>Accuracy ±1 nm Precision ≤0.5 nm</td>
<td>Accuracy ±2 nm Precision ≤0.5 nm</td>
<td>—</td>
</tr>
<tr>
<td>Didymium solutions or glasses</td>
<td>—</td>
<td>—</td>
<td>Accuracy ±2 nm Precision ≤0.5 nm</td>
</tr>
</tbody>
</table>
[Note—Certified reference standards, traceable to the National Institute of Standards and Technology (NIST) (www.nist.gov) or to other recognized standards-setting organizations, are commercially available, and should be used where possible.]

For non-diode array instruments, wavelength accuracy and precision are determined over the operational range using at least six replicates measurements. For wavelength accuracy, the difference of the mean measured value to the certified value of the CRM must be within ±1 nm in the UV region (200–400 nm), and in the visible region (400–780 nm) and visible/NIR (near-infrared) region (400–900 nm) must be within ±2 nm.

For wavelength precision, the standard deviation of the mean value of the six wavelength measurements must not exceed 0.5 nm. For diode array instruments, only one wavelength accuracy measurement is required, and no precision determination needs to be performed, due to the non-mechanical design of the monochromator.

Establishment of Acceptance Limits (Wavelength)—Choice of Standards

For all Control of Wavelengths accuracy procedures, acceptance limits for adequate calibration are established by adding the expanded uncertainty of the CRM to the instrument vendor specification at the wavelength(s) required in a linear manner, and these values must lie within the values specified in Table 1. In the case where atomic line spectra are used, this expanded uncertainty of the CRM is deemed to be zero for this process, i.e., the limit simply becomes the instrument vendor specification.¹

**ATOMIC LINE SPECTRA**

This procedure is described as the primary application because the emission lines produced from a discharge lamp are characteristic of the source element and, as a fundamental physical standard, these wavelengths have been measured with an uncertainty of NMT ±0.01 nm. In solution spectrometry, the wavelength accuracy required rarely exceeds 1.0 nm. For these reasons, the atomic line standard values are cited without uncertainty.

The arc of the atomic emission source, or its image, needs to be located on the same optical path as the image of the primary light source of the spectrometer; thus, it can be used only in spectrometers that can be operated in a single-beam intensity mode and practically should be implemented only on a system designed to accommodate these sources, e.g., as an accessory.

A commonly employed low-pressure mercury lamp has a number of intense lines that cover a large part of the UV and visible spectra. Two deuterium lines from the source at 486.0 and 656.1 nm often are used by manufacturers as an internal calibration check and can be used for diagnostic purposes, as can the Xe line at 260.6 nm, if appropriate (see Table 2).²

<table>
<thead>
<tr>
<th>Element</th>
<th>nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hg</td>
<td>296.7</td>
</tr>
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<td>Hg</td>
<td>313.2</td>
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<td>D₂</td>
<td>546.1</td>
</tr>
<tr>
<td>Hg</td>
<td>577.0</td>
</tr>
<tr>
<td>Hg</td>
<td>579.1</td>
</tr>
<tr>
<td>D₂</td>
<td>656.1</td>
</tr>
</tbody>
</table>

[Rare earth oxide solutions prepared by dissolution in acid media. The most frequently used is holmium oxide in perchloric acid.³ Holmium oxide solution has been internationally accepted as an intrinsic wavelength standard, and suitable CRMs are available commercially.⁴]

² The rounded values are taken from ASTM Standard E275.
⁴ CRMs produced under appropriate control, which may be demonstrated by accreditation to ISO/EC 17025 and/or ISO Guide 34 (ISO 17034).
The observed peak maxima are determined using the normal scan mode on the spectrometer. The peak maxima for a 4% (m/v) solution of holmium oxide in perchloric acid at 1.0-nm SBW and a path length of 1 cm are shown in Table 3.

Table 3. Recommended Peak Maxima from a 4% Solution of Holmium Oxide in Perchloric Acid for Wavelength Calibration Purposes

<table>
<thead>
<tr>
<th>nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>241.1</td>
</tr>
<tr>
<td>249.9</td>
</tr>
<tr>
<td>278.1</td>
</tr>
<tr>
<td>287.2</td>
</tr>
<tr>
<td>333.5</td>
</tr>
<tr>
<td>345.4</td>
</tr>
<tr>
<td>361.3</td>
</tr>
<tr>
<td>385.6</td>
</tr>
<tr>
<td>416.3</td>
</tr>
<tr>
<td>451.4</td>
</tr>
<tr>
<td>467.8</td>
</tr>
<tr>
<td>485.2</td>
</tr>
<tr>
<td>536.6</td>
</tr>
<tr>
<td>640.5</td>
</tr>
</tbody>
</table>

[NOTE—Select the nearest wavelength(s) spanning the operational range required.]

If the operational range of the spectrometer lies outside the range 240–650 nm, other certified rare earth oxides or other solutions can be used if they are traceable to a national or international standard. Cerium is available as a traceable solution CRM in acidic media, e.g., in sulfuric acid. It has useful peak characteristics in the 200–300 nm region at approximately 201.1, 211.4, 222.6, 240.4, and 253.7 nm.

Didymium (a mixture of neodymium and praseodymium) is available as a traceable CRM both in solution and as a glass. Didymium is similar in preparation to the holmium materials and has useful peak characteristics in the 730–870-nm region, which may vary from melt to melt of the glass. Useful peaks are found in the didymium solution at approximately 731.6, 740.0, 794.1, 800.0, and 864.4 nm.

RARE EARTH GLASSES

This procedure uses glasses manufactured by fusing the appropriate rare earth oxide in a base glass matrix. The most frequently used is holmium, for which the reference wavelengths have been well defined. Although manufacturing can cause batch variation in these glasses, traceable CRMs are commercially available and can be used. Typical values for a holmium glass using a 1.0-nm SBW are the following: 241.5, 279.2, 287.5, 333.8, 360.9, 418.8, 445.8, 453.7, 460.2, 536.5, and 637.7 nm.

Control of Absorbance

To establish the transmittance accuracy, precision, and linearity of a given system, it is necessary to verify the absorbance accuracy of a system over its intended operational range by selection and use of the following procedures as appropriate for the wavelength and absorbance ranges required.

The control of absorbance OQ must include at least one assessment at each wavelength range in the 0–2.00 A region. If absorbance >2.00 A is to be used for quantitation, then control of absorbance must also be evaluated in the >2.00 A range.

Establishment of Acceptance Limits (Absorbance)—Choice of Standards

For all Control of Absorbance procedures, acceptance limits for adequate calibration are established by adding the expanded uncertainty of the CRM to the instrument vendor specification at the wavelength(s) and absorbance levels required in a linear manner, and these values must lie within the values specified in Table 4.

[NOTE—CRMs, traceable to NIST Standard Reference Materials (SRMs), or other international or national standards, are commercially available and should be used where possible.]

Control of Photometric Response (Linearity)

Verification of photometric response (linearity) is required, and it should be evaluated using a standard type appropriate for the wavelength(s) required, where at least three different absorbance levels appropriate to and spanning the required operational range are measured. The options and acceptance criteria are summarized in Table 4.

[NOTE—Given the above requirement, it is not necessary to calculate the correlation coefficient of the standard response to demonstrate linearity; just demonstrate that at the different absorbance levels selected the acceptance limits have been met.]
Table 4. Available CRMs for the Control of Absorbance

<table>
<thead>
<tr>
<th>Absorbance Range</th>
<th>Standard</th>
<th>UV (200–400 nm)</th>
<th>Vis (400–780 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–1 A</td>
<td>Certified nicotinic acid solution</td>
<td>6–24 mg/L. Evaluate at 213 and 261 nm. Accuracy ±0.010 A, Precision ±0.005 A.</td>
<td>—</td>
</tr>
<tr>
<td>0–1 A</td>
<td>Certified potassium dichromate (K₂Cr₂O₇) solution</td>
<td>20–60 mg/L. Evaluate at 235, 257, 313, and 350 nm. Accuracy ±0.010 A, Precision ±0.005 A.</td>
<td>600 mg/L. Evaluate at 430 nm. Accuracy ±0.010 A, Precision ±0.005 A.</td>
</tr>
<tr>
<td>0–1 A</td>
<td>Certified neutral density glass filters</td>
<td>—</td>
<td>Evaluate at 440, 465, 546.1, 590, and 635 nm. Accuracy ±0.008 A, Precision ±0.005 A.</td>
</tr>
<tr>
<td>0–1 A</td>
<td>Certified metal-on-fused-silica filters</td>
<td>Evaluate at 250, 280, 340, 360, and 400 nm. Accuracy ±0.010 A, Precision ±0.005 A.</td>
<td>Evaluate at 465, 500, 546.1, 590, and 635 nm. Accuracy ±0.008 A, Precision ±0.005 A.</td>
</tr>
<tr>
<td>1–3 A</td>
<td>Certified potassium dichromate (K₂Cr₂O₇) solution</td>
<td>80–200 mg/L. Evaluate at 235, 257, 313, and 350 nm. Accuracy ±1%</td>
<td>—</td>
</tr>
<tr>
<td>1–3 A</td>
<td>Certified neutral density glass filters</td>
<td>—</td>
<td>Evaluate at 440, 465, 546.1, 590, and 635 nm. Accuracy ±0.008 A, Precision ±0.005 A.</td>
</tr>
<tr>
<td>UV Photometric linearity</td>
<td>Certified nicotinic acid solutions</td>
<td>All concentrations meet accuracy of absorbance acceptance criteria.</td>
<td>—</td>
</tr>
<tr>
<td>Photometric linearity</td>
<td>Certified potassium dichromate (K₂Cr₂O₇) solutions</td>
<td>All concentrations meet accuracy of absorbance acceptance criteria.</td>
<td>—</td>
</tr>
<tr>
<td>Photometric linearity</td>
<td>Certified metal-on-fused-silica filters</td>
<td>At least three certified filters evaluated at 250, 280, 340, 360, or 400 nm. All filters meet accuracy of absorbance acceptance criteria.</td>
<td>—</td>
</tr>
<tr>
<td>Photometric linearity</td>
<td>Certified neutral-density glass filters</td>
<td>—</td>
<td>At least three certified filters evaluated. All filters meet accuracy of absorbance acceptance criteria.</td>
</tr>
</tbody>
</table>

[NOTE—Select the appropriate absorbance CRMs spanning the operational range(s) required.]

ACIDIC NICOTINIC ACID SOLUTIONS IN 0.1 N HYDROCHLORIC ACID

In the 0–24 mg/L range, nicotinic acid solutions provide reference values of up to 1.0 A at one of the certified values of 213 or 261 nm. These solutions are available as CRMs, in the 0–60 mg/L range, providing reference values up to 2.5 A. Using nicotinic acid solutions, the absorbance accuracy must be ±0.010 A (ERR 1-Jan-2020). (for values below 1.00 A); ±0.008 A (ERR 1-Jan-2020).

The absorbance precision can be determined as the standard deviation from the mean of at least six replicate measurements. These sets of measurements should be performed, i.e., repeated at both the upper and lower absorbance values of the operational range. In each case, absorbance deviations from the mean must not exceed ±0.005 A, (for values below 1.0 A).

ACIDIC POTASSIUM DICHROMATE SOLUTIONS IN 0.001 M PERCHLORIC ACID

In the 0–200 mg/L range, potassium dichromate solutions provide reference values of up to 3.0 absorbance units at one of the certified values of 235, 257, 313, or 350 nm. These solutions are available as CRMs or can be prepared according to NIST from appropriate available SRMs, e.g., SRM 935x, or SRM 136x (where x can be any letter from “a” to “z”). Using potassium dichromate solutions, the absorbance accuracy must be ±1% A, (for values above 1.00 A) or ±0.010 A, (for values below 1.00 A).

The absorbance precision can be determined as the standard deviation from the mean of at least six replicate measurements. These sets of measurements should be performed, i.e., repeated at both the upper and lower absorbance values of the operational range. In each case, absorbance deviations from the mean must not exceed ±0.5% A, (for values above 1.0 A) or ±0.005 A, (for values below 1.0 A).
NEUTRAL-DENSITY GLASS FILTERS

These gray glass filters are manufactured from doped glass and have a nominally flat spectrum in the region of the calibration wavelengths. They provide reference values of up to 3.5 absorbance units at the certified values of 440, 465, 546.1, 590, and 635 nm. These filters are available as CRMs that are traceable to NIST SRM 930e, 1930, and 2930. Other certified standard solutions or optical filters can be used if they are traceable to a national or international standard. Using gray glass filters, the absorbance accuracy must be $\pm 0.8\% A_\lambda$ (for values above 1.00 $A_\lambda$) or $\pm 0.008 A_\lambda$ (for values below 1.00 $A_\lambda$). The absorbance precision can be determined as the standard deviation from the mean of at least six replicate measurements. The absorbance deviation from the mean must not exceed $\pm 0.5\% A$ (for values above 1.0 $A$) or $\pm 0.005 A_\lambda$ (for values below 1.0 $A_\lambda$).

Metal-on-Fused-Silica Filters

These neutral density filters are manufactured by deposition of a metallic film on a silica substrate and have a substantially flat spectrum in the region of the calibration wavelengths. They provide reference values of up to 2 $A$ at the usually certified values of 250, 280, 340, 360, and 400 nm in the UV range, and reference values of 465, 500, 546.1, 590, and 635 nm in the visible range. These filters are available as CRMs that are traceable to NIST SRM 2031x (where $x = "a"$ to "z"), or to other national or international standards. In the UV range, the absorbance accuracy must be $\pm 1\% A_\lambda$ (for values above 1.00 $A_\lambda$) or $\pm 0.01 A_\lambda$ (for values below 1.00 $A_\lambda$). In the visible range, the absorbance accuracy must be $\pm 0.8\% A_\lambda$ (for values above 1.00 $A_\lambda$) or $\pm 0.008 A_\lambda$ (for values below 1.00 $A_\lambda$). The absorbance precision can be determined as the standard deviation from the mean of at least six replicate measurements at the upper and lower absorbance values of the operational range. The standard deviation from the mean must not exceed $\pm 0.5\% A_\lambda$ (for values above 1.00 $A_\lambda$) or $\pm 0.005 A_\lambda$ (for values below 1.00 $A_\lambda$) in both the UV and visible ranges.

Estimation of the Limit of Stray Light (Stray Radiant Energy)

Although the measurement of absorbance or transmittance is a ratio measurement of intensities and therefore theoretically independent of monochromatic source intensity, practical measurements are affected by the presence of unwanted radiation called "stray radiant energy" or "stray light". In addition, the adverse effect of stray light increases with aging of optical components and lamps in a spectrometer. The effects are greater at the extremes of detector and lamp operational ranges. The limit of stray light OQ must include evaluation at one or more UV wavelengths, by selection of appropriate material(s) shown in Table 5 to span the UV operational range required. In the visible region, i.e., above 400 nm, stray light does not need to be evaluated.

[Note—Published stray light specifications for a given spectrometer must be verified in the OQ.]

Analyzers must choose and use the appropriate reference(s) to monitor the level of stray light as part of PQ. Stray light can be detected at a given wavelength with a suitable liquid filter by either of the two procedures (A or B) detailed below.

[Note—If measurements are being performed in the 250–330 nm region, on a spectrometer using individual sources for the UV and visible regions of the spectrum, then an additional PQ verification using acetone should be performed.]

These solutions are available as CRMs or can be prepared at the concentrations shown in Table 5 by using reagent-grade materials.

Table 5. Spectral Ranges of Selected Materials for Monitoring Stray Light

<table>
<thead>
<tr>
<th>Recommended Wavelength (nm)</th>
<th>Spectral Range (nm)</th>
<th>Liquid or Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>198</td>
<td>190–210</td>
<td>Aqueous potassium chloride (12 g/L)</td>
</tr>
<tr>
<td>220</td>
<td>210–270</td>
<td>Aqueous sodium iodide or potassium iodide (10 g/L)</td>
</tr>
<tr>
<td>300</td>
<td>250–330</td>
<td>Acetone</td>
</tr>
<tr>
<td>340</td>
<td>300–400</td>
<td>Aqueous sodium nitrite (50 g/L)</td>
</tr>
</tbody>
</table>

Procedure A

The aim of this procedure is to produce the differential spectrum resulting from the subtraction of a spectrum produced by a 5-mm path length cell from that of a 10-mm cell, both filled with the same filter solution. This spectrum will contain a peak maximum absorbance value, and analysts can calculate the stray light value from the observed maximum absorbance using the formula:

$$s_\lambda = 0.25 \times 10^{-2 A_\lambda}$$

$s_\lambda$ = stray light value, in transmittance ($T$)

$A_\lambda$ = observed maximum absorbance

This procedure requires the 10-mm cell measurement to be subtracted from the 5-mm cell, both filled with the same cut-off solution filter. This measurement can be achieved by either using the physical capabilities of the spectrometer, i.e., using the double-beam capability, with the 5-mm cell as the reference, or by mathematically subtracting the spectra of the 10-mm cell from the 5-mm cell, chronologically produced by sequential measurement of both the 5- and 10-mm cells, using the single-sample beam, with the spectrometer referenced to air (blank holder).
**Control of Resolution**

If accurate absorbance measurements must be made on benzenoid compounds or other compounds with sharp absorption bands (natural half-bandwidths of <15 nm), the SBW of the spectrometer used should not be greater than 1/8th the natural half-bandwidth of the compound’s absorption; i.e., this equates to a spectrometer with a SBW of 2 nm or less.

Determine the resolution of the spectrometer in the UV region by using the following procedure. Using UV-grade n-hexane as the reference, measure the absorbance of a 0.020% (v/v) solution of toluene in UV-grade n-hexane at the maximum, about 269 nm, and minimum, about 266 nm. The ratio of the absorbance at the maximum to the absorbance at the minimum will typically fall in the 1.0–2.6 range.

For most compendial quantitative purposes, a SBW of 2 nm or less is sufficient, and the Acceptance criteria for the ratio is NLT 1.3.

**Performance Qualification**

The purpose of PQ is to determine that the instrument is capable of meeting the user’s requirements for all the parameters that may affect the quality of the measurement and to ensure that it will function properly over extended periods of time.

**PROCEDURE**

With few exceptions, compendial spectrometric tests and assays call for comparison against a USP Reference Standard. This helps ensure measurement under identical conditions for the test specimen and the reference substance. These conditions could include wavelength setting, SBW selection, cell placement and correction, and transmittance levels. Cells that exhibit identical transmittance at a given wavelength may differ considerably in transmittance at other wavelengths. Appropriate cell corrections should be established and used where required.

Comparisons of a test specimen with a reference standard are best made at a peak of spectral absorption for the compound concerned. Assays that prescribe spectrometry give the commonly accepted wavelength for peak spectral absorption of the substance in question. Different spectrometers may show minor variation in the apparent wavelength of this peak. Good practice demands that comparisons be made at the wavelength at which peak absorption occurs. Should this differ by >±1 nm (in the range 200–400 nm) or ±2 nm (in the range 400–800 nm) from the wavelength specified in the individual monograph, recalibration of the instrument may be indicated.

The expressions “similar preparation” and “similar solution” as used in tests and assays involving spectrometry indicate that the reference comparator, generally a USP Reference Standard, should be prepared and observed in an identical manner for all practical purposes to that used for the test specimen. Usually when analysts make up the solution of the specified reference standard, they prepare a solution of about (i.e., within 10%) of the desired concentration, and they calculate the absorptivity on the basis of the exact amount weighed out. If a previously dried specimen of the reference standard has not been used, the absorptivity is calculated on the anhydrous basis. The expressions “concomitantly determine” and “concomitantly measure” as used in tests and assays involving spectrometry indicate that the absorbances of both the solution containing the test specimen and the solution containing the reference specimen, relative to the specified test blank, must be measured in immediate succession.

**Sample Solution Preparation**

For determinations using UV or visible spectrometry, the specimen generally is dissolved in a solvent. Unless otherwise directed in the monograph, analysts make determinations at room temperature using a path length of 1 cm. Many solvents are suitable for these ranges, including water, alcohols, lower hydrocarbons, ethers, and dilute solutions of strong acids and alkalis. Precautions should be taken to use solvents that are free from contaminants that absorb in the spectral region under examination. For the solvent, analysts typically should use water-free methanol or alcohol or alcohol denatured by the addition of methanol but without benzene or other interfering impurities. Solvents of special spectrometric quality, guaranteed to be free from contaminants, are available commercially from several sources. Some other analytical reagent-grade organic solvents may contain traces of impurities that absorb strongly in the UV region. New lots of these solvents should be checked for their transparency, and analysts should take care to use the same lot of solvent for preparation of the test solution, the standard solution, and the blank. The best practice is to use solvents that have NLT 40% transmittance (39.9% T = 0.399 A) at the wavelength of interest.

Assays in the visible region usually call for concomitantly comparing the absorbance produced by the assay preparation with that produced by a standard preparation containing approximately an equal quantity of a USP Reference Standard. In some situations, analysts can omit the use of a reference standard (e.g., when spectrometric assays are made with routine frequency) when a suitable standard curve is available and is prepared with the appropriate USP Reference Standard, and when the
substance assayed conforms to the Beer–Lambert law within the range of about 75%–125% of the final concentration used in the assay. Under these circumstances, the absorbance found in the assay may be interpolated on the standard curve, and the assay result can be calculated. Such standard curves should be confirmed frequently and always when a new spectrometer or new lots of reagents are put into use.

VERIFICATION AND VALIDATION

Verification

Current Good Manufacturing Practices regulations [21 CFR 211.194(a)(2)] indicate that users of analytical procedures described in USP–NF are not required to validate these procedures if provided in a monograph. Instead, they simply must verify their suitability under actual conditions of use.

The objective of a UV-Vis procedure verification is to demonstrate the suitability of a test procedure under actual conditions of use. Performance characteristics that verify the suitability of a UV-Vis procedure are similar to those required for any analytical procedure. A discussion of the applicable general principles is found in Verification of Compendial Procedures (1226). Verification is usually performed using a reference material and a well-defined matrix. Verification of compendial UV-Vis procedures includes at minimum the execution of the validation parameters for specificity, accuracy, precision, and quantitation limit, when appropriate, as indicated in Validation.

Validation

Validation is required when a UV-Vis method is intended for use as an alternative to the official procedure for testing an official article or when no official procedure exists in the current USP–NF.

The objective of UV-Vis method validation is to demonstrate that the measurement is suitable for its intended purpose, including quantitative determination of the main component in a drug substance or a drug product (Category I assays), quantitative determination of impurities or limit tests (Category II), and identification tests (Category IV). For dissolution procedures, see The Dissolution Procedure: Development and Validation (1092). Depending on the category of the test (see Validation of Compendial Procedures (1225), Table 2), the analytical method validation process for UV-Vis requires testing for linearity, range, accuracy, specificity, precision, detection limit, or quantitation limit. These analytical performance characteristics apply to externally standardized procedures and those that use standard additions.

Chapter (1225) provides definitions and general guidance on analytical procedures validation without indicating specific validation criteria for each characteristic. The intention of the following sections is to provide the user with specific validation criteria that represent the minimum expectations for this technology. For each particular application, tighter criteria may be needed in order to demonstrate suitability for the intended use.

ACCURACY

For Category I, II, and III procedures, accuracy can be determined by conducting recovery studies with the appropriate matrix spiked with known concentrations of the analyte. Analysts also can compare assay results obtained using the UV-Vis procedure under validation to those from an established analytical procedure.

Validation criteria: 98.0%–102.0% mean recovery for the drug substances, 95.0%–105.0% mean recovery for the drug product assay, and 80.0%–120.0% mean recovery for the impurity analysis. These criteria are met throughout the intended range.

Precision

REPEATABILITY

The repeatability of the analytical procedure is assessed by measuring the concentrations of six independently prepared sample solutions at 100% of the assay test concentration. Alternatively, it can be assessed by measuring the concentrations of three replicates of three separate sample solutions at different concentrations. The three concentrations should be close enough so that the repeatability is constant across the concentration range. If this is done, the repeatability at the three concentrations is pooled for comparison to the acceptance criteria.

Validation criteria: The relative standard deviation is NMT 1.0% for the drug substance assay, NMT 2.0% for the drug product assay, and NMT 15.0%–20.0% for the impurity analysis.

INTERMEDIATE PRECISION

The effect of random events on the analytical precision of the method must be established. Typical variables include performing the analysis on different days, using different instrumentation, and/or having the method performed by two or more analysts. At a minimum, any combination of at least two of these factors totaling six experiments will provide an estimation of intermediate precision.

Validation criteria: The relative standard deviation is NMT 1.5% for the drug substance assay, NMT 3.0% for the drug product assay, and NMT 15.0%–25.0% for the impurity analysis.
SPECIFICITY

In UV-Vis measurements, specificity is ensured by the use of a reference standard wherever possible and is demonstrated by the lack of interference from other components present in the matrix.

DETECTION LIMIT

The detection limit (DL) can be estimated by calculating the standard deviation of NLT 6 replicate measurements of a blank solution and multiplying by 3.3. Alternatively, the standard deviation can be determined from the error of the intercept from a calibration curve or by determining that the signal-to-noise ratio is >3.3. The estimated DL must be confirmed by analyzing samples at the calculated concentration.

QUANTITATION LIMIT

The quantitation limit (QL) can be estimated by calculating the standard deviation of NLT 6 replicate measurements of a blank solution and multiplying by 10. Alternatively, the standard deviation can be determined from the error of the intercept from a calibration curve or by determining that the signal-to-noise ratio is >10. Measurement of a test solution prepared from a representative sample matrix spiked at the required QL concentration must be performed to confirm sufficient sensitivity and adequate precision. The observed signal-to-noise ratio at the required QL should be >10. [Note—A suitable procedure for measuring the signal-to-noise ratio is given in ASTM 1657-98 (2011) Standard Practice for the Testing of Variable-Wavelength Photometric Detectors Used in Liquid Chromatography.]

Validation criteria: For the estimated limit of quantitation to be considered valid, the measured concentration must be accurate and precise at a level ≤50% of the specification.

LINEARITY

A linear relationship between the analyte concentration and UV-Vis response must be demonstrated by preparation of NLT 5 standard solutions at concentrations encompassing the anticipated concentration of the test solution. The standard curve is then evaluated using appropriate statistical methods such as a least-squares regression. Deviation from linearity results from either instrumental or sample factors, or both, and can be reduced to acceptable levels by reducing the analyte concentration and thereby the associated absorbance values. The (Pearson) correlation coefficient, \( r \), measures the strength and direction of the association between two variables (\( x \) and \( y \)), in this instance, concentration and absorbance. The coefficient of determination, \( r^2 \), is a measure of the fraction of the data’s variation that is adequately modeled and not a measure of linearity. Linearity depends on the standard error of the calibration equation (and hence the reference procedure) and on the range of the calibration data. Thus, although values very near 1.00, such as 0.99 or greater, typically indicate a linear relationship, lower values do not distinguish between nonlinearity and variability. Validation criteria: The coefficient of determination, \( r^2 \), must be NLT 0.995 for Category I assays and NLT 0.99 for Category II quantitative tests. Visual inspection of the residual plots should not reveal any significant pattern.

RANGE

The operational range of an analytical instrument (and the analytical procedure as a whole) is the interval between the upper and lower concentrations (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the instrumental response function has a suitable level of precision, accuracy, and linearity. Validation criteria: For Category I tests, the validation range for 100.0% centered acceptance criteria is 80.0%–120.0%. For non-centered acceptance criteria, the validation range is 10.0% below the lower limit to 10.0% above the upper limit. For content uniformity, the validation range is 70.0%–130.0%. For Category II tests, the validation range covers 50.0%–120.0% of the acceptance criteria.

ROBUSTNESS

This parameter is evaluated during method development. The reliability of an analytical measurement is demonstrated by deliberate changes to experimental parameters. For UV-Vis, this can include measuring the stability of the analyte under specified storage conditions, varying pH, and adding possible interfering species, to list a few examples. Robustness is determined concurrently using a suitable design for the experimental procedure.

INDIRECT MEASUREMENT REQUIREMENTS

For certain UV-Vis procedures, chromogenic reactions are employed. Generally, the requirements for the analytical performance characteristics are used. In some instances, the required accuracy and precision criteria for the direct measurements may not be achievable. Under these circumstances, the accuracy and precision requirements can be widened by as much as 50%. Any such widening must be justified on scientific grounds and with documented evidence. It may be necessary to increase the amount of replication required to produce a scientifically sound reportable value.
UNIFORMITY OF DOSAGE UNITS

This general chapter is harmonized with the corresponding texts of the European Pharmacopoeia and the Japanese Pharmacopoeia. Portions of the general chapter text that are national USP text, and are not part of the harmonized text, are marked with symbols (*) to specify this fact.

[Note—In this chapter, unit and dosage unit are synonymous.]

To ensure the consistency of dosage units, each unit in a batch should have a drug substance content within a narrow range around the label claim. Dosage units are defined as dosage forms containing a single dose or a part of a dose of drug substance in each unit. The uniformity of dosage units specification is not intended to apply to suspensions, emulsions, or gels in unit-dose containers intended for external, cutaneous administration.

The term “uniformity of dosage unit” is defined as the degree of uniformity in the amount of the drug substance among dosage units. Therefore, the requirements of this chapter apply to each drug substance being comprised in dosage units containing one or more drug substances, unless otherwise specified elsewhere in this Pharmacopeia.

The uniformity of dosage units can be demonstrated by either of two methods, Content Uniformity or *Weight, Variation (see Table 1). The test for Content Uniformity of preparations presented in dosage units is based on the assay of the individual content of drug substance(s) in a number of dosage units to determine whether the individual content is within the limits set. The Content Uniformity method may be applied in all cases.

The test for *Weight, Variation is applicable for the following dosage forms:

<table>
<thead>
<tr>
<th>Dosage Form Type</th>
<th>Type</th>
<th>Subtype</th>
<th>Dose &amp; Ratio of Drug Substance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tablets</td>
<td>Uncoated</td>
<td>WV</td>
<td>CU</td>
</tr>
<tr>
<td></td>
<td>Coated</td>
<td>WV</td>
<td>CU</td>
</tr>
<tr>
<td></td>
<td>Others</td>
<td>CU</td>
<td>CU</td>
</tr>
<tr>
<td>Capsules</td>
<td>Hard</td>
<td>WV</td>
<td>CU</td>
</tr>
<tr>
<td></td>
<td>Soft</td>
<td>Suspension, emulsion, or gel</td>
<td>CU</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Solutions</td>
<td>WV</td>
</tr>
<tr>
<td>Solids in single-unit containers</td>
<td>Single component</td>
<td>WV</td>
<td>WV</td>
</tr>
<tr>
<td></td>
<td>Multiple components</td>
<td>Solution freeze-dried in final container</td>
<td>WV</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Others</td>
<td>CU</td>
</tr>
<tr>
<td>Solutions in unit-dose containers (and into soft capsules)</td>
<td>WV</td>
<td>WV</td>
<td></td>
</tr>
<tr>
<td>Others</td>
<td></td>
<td>CU</td>
<td>CU</td>
</tr>
</tbody>
</table>

The test for Content Uniformity is required for all dosage forms not meeting the above conditions for the *Weight, Variation test.

Table 1. Application of Content Uniformity (CU) and Weight Variation (WV) Tests for Dosage Forms

CONTENT UNIFORMITY

Select not fewer than 30 units, and proceed as follows for the dosage form designated.

1 European Pharmacopoeia and Japanese Pharmacopoeia text not accepted by the United States Pharmacopoeia: Alternatively, products listed in item (4) above that do not meet the 25 mg/25% threshold limit may be tested for uniformity of dosage units by Mass Variation instead of the Content Uniformity test if the concentration relative standard deviation (RSD) of the drug substance in the final dosage units is not more than 2%, based on process validation data and development data, and if there has been regulatory approval of such a change. The concentration RSD is the RSD of the concentration per dosage unit (w/w or w/v), where concentration per dosage unit equals the assay result per dosage unit divided by the individual dosage unit weight. See the RSD formula in Table 2.
Where different procedures are used for assay of the preparation and for the Content Uniformity test, it may be necessary to establish a correction factor to be applied to the results of the latter.

**Solid Dosage Forms**

Assay 10 units individually using an appropriate analytical method. Calculate the acceptance value (see Table 2).

**Liquid or Semi-Solid Dosage Forms**

Assay 10 units individually using an appropriate analytical method. Carry out the assay on the amount of well-mixed material that is removed from an individual container in conditions of normal use, and express the results as delivered dose. Calculate the acceptance value (see Table 2).

**Calculation of Acceptance Value**

Calculate the acceptance value by the formula:

\[
M - \bar{X} + ks
\]

in which the terms are as defined in Table 2.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Definition</th>
<th>Conditions</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \bar{X} )</td>
<td>Mean of individual contents ( (\chi_1, \chi_2, \ldots, \chi_n) ), expressed as a percentage of the label claim</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \chi_1, \chi_2, \ldots, \chi_n )</td>
<td>Individual contents of the units tested, expressed as a percentage of the label claim</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( n )</td>
<td>Sample size (number of units in a sample)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( k )</td>
<td>Acceptability constant</td>
<td>If ( n = 10 ), then ( k = ) 2.4 If ( n = 30 ), then ( k = ) 2.0</td>
<td></td>
</tr>
</tbody>
</table>
| \( s \) | Sample standard deviation | \[
\sum_{i=1}^{n} \left( \chi_i - \bar{X} \right)^2 / (n - 1)
\] |
| RSD | Relative standard deviation (the sample standard deviation expressed as a percentage of the mean) | 100s/\( \bar{X} \) |
| \( M \) (case 1) to be applied when \( T \leq 101.5 \) | Reference value | If 98.5% \( \leq \bar{X} \leq 101.5% \), then \( M = \bar{X} \) (AV = ks) If \( \bar{X} < 98.5% \), then \( M = 98.5% \) (AV = 98.5 - \( \bar{X} + ks \)) If \( \bar{X} > 101.5% \), then \( M = 101.5% \) (AV = \( \bar{X} - 101.5 + ks \)) |
| \( M \) (case 2) to be applied when \( T > 101.5 \) | Reference value | If 98.5% \( \leq \bar{X} \leq T \), then \( M = \bar{X} \) (AV = ks) If \( \bar{X} < 98.5% \), then \( M = 98.5% \) (AV = 98.5 - \( \bar{X} + ks \)) If \( \bar{X} > T \), then \( M = T% \) (AV = \( \bar{X} - T + ks \)) |
| Acceptance value (AV) | General formula: | \[
M - \bar{X} + ks
\]
(Calculations are specified above for the different cases.) |
| L1 | Maximum allowed acceptance value | L1 = 15.0 unless otherwise specified |

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Table 2 (continued)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Definition</th>
<th>Conditions</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>L2</td>
<td>Maximum allowed range for deviation of each dosage unit tested from the calculated value of M</td>
<td>On the low side, no dosage unit result can be less than $[1 - (0.01)(L2)]M$, while on the high side, no dosage unit result can be greater than $[1 + (0.01)(L2)]M$. (This is based on an L2 value of 25.0.)</td>
<td>$L2 = 25.0$ unless otherwise specified</td>
</tr>
</tbody>
</table>

**WEIGHT, VARIATION**

Carry out an assay for the drug substance(s) on a representative sample of the batch using an appropriate analytical method. This value is result $A$, expressed as percentage of label claim (see Calculation of Acceptance Value). Assume that the concentration (weight of drug substance per weight of dosage unit) is uniform. Select not fewer than 30 dosage units, and proceed as follows for the dosage form designated.

**Uncoated or Film-Coated Tablets**

Accurately weigh 10 tablets individually. Calculate the content, expressed as percentage of label claim, of each tablet from the *weight* of the individual tablet and the result of the *Assay*. Calculate the acceptance value.

**Hard Capsules**

Accurately weigh 10 capsules individually, taking care to preserve the identity of each capsule. Remove the contents of each capsule by a suitable means. Accurately weigh the emptied shells individually, and calculate for each capsule the net *weight* of its contents by subtracting the *weight* of the shell from the respective gross *weight*. Calculate the drug substance content of each capsule from the *net weight* of the individual capsule *content*, and the result of the *Assay*. Calculate the acceptance value.

**Soft Capsules**

Accurately weigh 10 intact capsules individually to obtain their gross *weights*, taking care to preserve the identity of each capsule. Then cut open the capsules by means of a suitable clean, dry cutting instrument such as scissors or a sharp open blade, and remove the contents by washing with a suitable solvent. Allow the occluded solvent to evaporate from the shells at room temperature over a period of about 30 minutes, taking precautions to avoid uptake or loss of moisture. Weigh the individual shells, and calculate the net contents. Calculate the drug substance content in each capsule from the *weight* of product removed from the individual capsules and the result of the *Assay*. Calculate the acceptance value.

**Solid Dosage Forms Other Than Tablets and Capsules**

Proceed as directed for *Hard Capsules*, treating each unit as described therein. Calculate the acceptance value.

**Liquid Dosage Forms**

Accurately weigh the amount of liquid that is removed from each of 10 individual containers in conditions of normal use. If necessary, compute the equivalent volume after determining the density. Calculate the drug substance content in each container from the mass of product removed from the individual containers and the result of the *Assay*. Calculate the acceptance value.

**Calculation of Acceptance Value**

Calculate the acceptance value as shown in *Content Uniformity*, except that the individual contents of the units are replaced with the individual estimated contents defined below.

\[
\chi_1, \chi_2, \ldots, \chi_n = \text{individual estimated contents of the units tested, where } \chi = w \times A/W
\]

\[
w_1, w_2, \ldots, w_n = \text{individual } *\text{weights}, \text{ of the units tested}
\]

\[
A = \text{content of drug substance } (\% \text{ of label claim}) \text{ obtained using an appropriate analytical method}
\]
\[ W = \text{mean of individual weights} \]

\[ (w_1, w_2, ..., w_n) \]

**CRITERIA**

Apply the following criteria, unless otherwise specified.

**Solid, Semi-Solid, and Liquid Dosage Forms**

The requirements for dosage uniformity are met if the acceptance value of the first 10 dosage units is less than or equal to L1%. If the acceptance value is > L1%, test the next 20 units, and calculate the acceptance value. The requirements are met if the final acceptance value of the 30 dosage units is \[ \leq L1\% \], and no individual content of *any* dosage unit is less than \[ [1 - (0.01)(L2)]M \] nor more than \[ [1 + (0.01)(L2)]M \] as specified in the *Calculation of Acceptance Value under Content Uniformity* or under *Weight, Variation*. Unless otherwise specified, L1 is 15.0 and L2 is 25.0.

**〈921〉 WATER DETERMINATION**

Many Pharmacopeial articles either are hydrates or contain water in adsorbed form. As a result, the determination of the water content is important in demonstrating compliance with the Pharmacopeial standards. Generally one of the methods given below is called for in the individual monograph, depending upon the nature of the article. In rare cases, a choice is allowed between two methods. When the article contains water of hydration, *Method I (Titrimetric)*, *Method II (Azeotropic)*, or *Method III (Gravimetric)* is employed, as directed in the individual monograph, and the requirement is given under the heading *Water*.

The heading *Loss on Drying* (see *Loss on Drying (731)*) is used in those cases where the loss sustained on heating may be not entirely water.

**METHOD I (TITRIMETRIC)**

Determine the water by *Method Ia*, unless otherwise specified in the individual monograph.

**Method Ia (Direct Titration)**

**PRINCIPLE**

The titrimetric determination of water is based upon the quantitative reaction of water with an anhydrous solution of sulfur dioxide and iodine in the presence of a buffer that reacts with hydrogen ions.

In the original titrimetric solution, known as Karl Fischer Reagent, the sulfur dioxide and iodine are dissolved in pyridine and methanol. The test specimen may be titrated with the *Reagent* directly, or the analysis may be carried out by a residual titration procedure. The stoichiometry of the reaction is not exact, and the reproducibility of a determination depends upon such factors as the relative concentrations of the *Reagent* ingredients, the nature of the inert solvent used to dissolve the test specimen, and the technique used in the particular determination. Therefore, an empirically standardized technique is used in order to achieve the desired accuracy. Precision in the method is governed largely by the extent to which atmospheric moisture is excluded from the system. The titration of water is usually carried out with the use of anhydrous methanol as the solvent for the test specimen. In some cases, other suitable solvents may be used for special or unusual test specimens. In these cases, the addition of at least 20% of methanol or other primary alcohol is recommended.

**APPARATUS**

Any apparatus may be used that provides for adequate exclusion of atmospheric moisture and determination of the endpoint. In the case of a colorless solution that is titrated directly, the endpoint may be observed visually as a change in color from canary yellow to amber. The reverse is observed in the case of a test specimen that is titrated residually. More commonly, however, the endpoint is determined electrometrically with an apparatus employing a simple electrical circuit that serves to impress about 200 mV of applied potential between a pair of platinum electrodes immersed in the solution to be titrated. At the endpoint of the titration a slight excess of the reagent increases the flow of current to between 50 and 150 microamperes for 30 s to 30 min, depending upon the solution being titrated. The time is shortest for substances that dissolve in the reagent. With some automatic titrators, the abrupt change in current or potential at the endpoint serves to close a solenoid-operated valve that controls the buret delivering the titrant. Commercially available apparatus generally comprises a closed system consisting of one or two automatic burets and a tightly covered titration vessel fitted with the necessary electrodes and a magnetic stirrer. The air in the system is kept dry with a suitable desiccant, and the titration vessel may be purged by means of a stream of dry nitrogen or current of dry air.

**REAGENT**

Prepare the Karl Fischer Reagent as follows. Add 125 g of iodine to a solution containing 670 mL of methanol and 170 mL of pyridine, and cool. Place 100 mL of pyridine in a 250-mL graduated cylinder, and, keeping the pyridine cold in an ice bath,
pass in dry sulfur dioxide until the volume reaches 200 mL. Slowly add this solution, with shaking, to the cooled iodine mixture. Shake to dissolve the iodine, transfer the solution to the apparatus, and allow the solution to stand overnight before standardizing. One mL of this solution when freshly prepared is equivalent to approximately 5 mg of water, but it deteriorates gradually; therefore, standardize it within 1 h before use, or daily if in continuous use. Protect from light while in use. Store any bulk stock of the reagent in a suitably sealed, glass-stoppered container, fully protected from light, and under refrigeration. For determination of trace amounts of water (less than 1%), it is preferable to use a Reagent with a water equivalency factor of not more than 2.0, which will lead to the consumption of a more significant volume of titrant.

A commercially available, stabilized solution of Karl Fischer type reagent may be used. Commercially available reagents containing solvents or bases other than pyridine or alcohols other than methanol may be used also. These may be single solutions or reagents formed in situ by combining the components of the reagents present in two discrete solutions. The diluted Reagent called for in some monographs should be diluted as directed by the manufacturer. Either methanol or other suitable solvent, such as ethylene glycol monomethyl ether, may be used as the diluent.

**TEST PREPARATION**

Unless otherwise specified in the individual monograph, use an accurately weighed or measured amount of the specimen under test estimated to contain 2–250 mg of water. The amount of water depends on the water equivalency factor of the Reagent and on the method of endpoint determination. In most cases, the minimum amount of specimen, in mg, can be estimated using the formula:

\[
\text{FCV/KF}
\]

in which \( F \) is the water equivalency factor of the Reagent, in mg per mL; \( C \) is the used volume, in percent, of the capacity of the buret; \( V \) is the buret volume, in mL; and \( KF \) is the limit or reasonable expected water content in the sample, in percent. \( C \) is generally between 30% and 100% for manual titration, and between 10% and 100% for the instrumental method endpoint determination.

[Note—It is recommended that the product of FCV be greater than or equal to 200 for the calculation to ensure that the minimum amount of water titrated is greater than or equal to 2 mg.]

Where the specimen under test is an aerosol with propellant, store it in a freezer for not less than 2 h, open the container, and test 10.0 mL of the well-mixed specimen. In titrating the specimen, determine the endpoint at a temperature of 10° or higher.

Where the specimen under test is capsules, use a portion of the mixed contents of not fewer than four capsules.

Where the specimen under test is tablets, use powder from not fewer than four tablets ground to a fine powder in an atmosphere of temperature and relative humidity known not to influence the results.

Where the monograph specifies that the specimen under test is hygroscopic, take an accurately weighed portion of the solid into the titration vessel, proceeding as soon as possible and taking care to avoid moisture uptake from the atmosphere. If the sample is constituted by a finite amount of solid as a lyophilized product or a powder inside a vial, use a dry syringe to inject an appropriate volume of methanol, or other suitable solvent, accurately measured, into a tared container, and shake to dissolve the specimen. Using the same syringe, remove the solution from the container and transfer it to a titration vessel prepared as directed for Procedure. Repeat the procedure with a second portion of methanol, or other suitable solvent, accurately measured, add this washing to the titration vessel, and immediately titrate. Determine the water content, in mg, of a portion of solvent of the same total volume as that used to dissolve the specimen and to wash the container and syringe, as directed for Standardization of Water Solution for Residual Titration, and subtract this value from the water content, in mg, obtained in the titration of the specimen under test. Dry the container and its closure at 100° for 3 h, allow to cool in a desiccator, and weigh. Determine the weight of specimen tested from the difference in weight from the initial weight of the container. When appropriate, the water may be desorbed or released from the sample by heat in an external oven connected with the vessel, to which it is transferred with the aid of an inert and dried gas such as pure nitrogen. Any drift due to the transport gas should be considered and corrected. Care should be taken in the selection of the heating conditions to avoid the formation of water coming from dehydration due to decomposition of the sample constituents, which may invalidate this approach.

**STANDARDIZATION OF THE REAGENT**

Place enough methanol or other suitable solvent in the titration vessel to cover the electrodes, and add sufficient Reagent to give the characteristic endpoint color, or 100 ± 50 microamperes of direct current at about 200 mV of applied potential. Purified Water, sodium tartrate dihydrate, a USP Reference Standard, or commercial standards with a certificate of analysis traceable to a national standard may be used to standardize the Reagent. The reagent equivalency factor, the recommended titration volume, buret size, and amount of standard to measure are factors to consider when deciding which standard and how much to use. For Purified Water or water standards, quickly add the equivalent of between 2 and 250 mg of water. Calculate the water equivalency factor, \( F \), in mg of water per mL of reagent:

\[
\frac{W}{V}
\]

in which \( W \) is the weight, in mg, of the water contained in the aliquot of standard used; and \( V \) is the volume, in mL, of the Reagent used in the titration. For sodium tartrate dihydrate, quickly add 20–125 mg of sodium tartrate dihydrate (C₆H₄Na₂O₆)
accurately weighed by difference, and titrate to the endpoint. The water equivalence factor \( F \), in mg of water per mL of reagent, is given by the formula:

\[
\frac{W}{V} \left( \frac{36.04}{230.08} \right)
\]

in which 36.04 is two times the molecular weight of water and 230.08 is the molecular weight of sodium tartrate dihydrate; \( W \) is the weight, in mg, of sodium tartrate dihydrate; and \( V \) is the volume, in mL, of the Reagent consumed in the second titration. Note that the solubility of sodium tartrate dihydrate in methanol is such that fresh methanol may be needed for additional titrations of the sodium tartrate dihydrate standard.

### Procedure

Unless otherwise specified, transfer enough methanol or other suitable solvent to the titration vessel, ensuring that the volume is sufficient to cover the electrodes (approximately 30–40 mL), and titrate with the Reagent to the electrometric or visual endpoint to consume any moisture that may be present. (Disregard the volume consumed, because it does not enter into the calculations.) Quickly add the Test Preparation, mix, and again titrate with the Reagent to the electrometric or visual endpoint. Calculate the water content of the specimen taken, in mg:

\[
SF
\]

in which \( S \) is the volume, in mL, of the Reagent consumed in the second titration; and \( F \) is the water equivalence factor of the Reagent.

### Method Ib (Residual Titration)

#### Principle

See the information given in the section Principle under Method Ia. In the residual titration, excess Reagent is added to the test specimen, sufficient time is allowed for the reaction to reach completion, and the unconsumed Reagent is titrated with a standard solution of water in a solvent such as methanol. The residual titration procedure is applicable generally and avoids the difficulties that may be encountered in the direct titration of substances from which the bound water is released slowly.

#### Apparatus, Reagent, and Test Preparation

Use Method Ia.

#### Standardization of Water Solution for Residual Titration

Prepare a Water Solution by diluting 2 mL of water with methanol or other suitable solvent to 1000 mL. Standardize this solution by titrating 25.0 mL with the Reagent, previously standardized as directed under Standardization of the Reagent. Calculate the water content, in mg per mL, of the Water Solution taken:

\[
\frac{VV}{25}
\]

in which \( V \) is the volume of the Reagent consumed, and \( F \) is the water equivalence factor of the Reagent. Determine the water content of the Water Solution weekly, and standardize the Reagent against it periodically as needed.

#### Procedure

Where the individual monograph specifies that the water content is to be determined by Method Ib, transfer enough methanol or other suitable solvent to the titration vessel, ensuring that the volume is sufficient to cover the electrodes (approximately 30–40 mL), and titrate with the Reagent to the electrometric or visual endpoint. Quickly add the Test Preparation, mix, and add an accurately measured excess of the Reagent. Allow sufficient time for the reaction to reach completion, and titrate the unconsumed Reagent with standardized Water Solution to the electrometric or visual endpoint. Calculate the water content of the specimen, in mg, taken:

\[
F(X' - XR)
\]

in which \( F \) is the water equivalence factor of the Reagent; \( X' \) is the volume, in mL, of the Reagent added after introduction of the specimen; \( X \) is the volume, in mL, of standardized Water Solution required to neutralize the unconsumed Reagent; and \( R \) is the ratio, \( V'/25 \) (mL Reagent/mL Water Solution), determined from the Standardization of Water Solution for Residual Titration.

### Method Ic (Coulometric Titration)

#### Principle

The Karl Fischer reaction is used in the coulometric determination of water. Iodine, however, is not added in the form of a volumetric solution but is produced in an iodide-containing solution by anodic oxidation. The reaction cell usually consists of a large anode compartment and a small cathode compartment that are separated by a diaphragm. Other suitable types of reaction...
cells (e.g., without diaphragms) may also be used. Each compartment has a platinum electrode that conducts current through the cell. Iodine, which is produced at the anode electrode, immediately reacts with water present in the compartment. When all the water has been consumed, an excess of iodine occurs, which usually is detected electrometrically, thus indicating the endpoint. Moisture is eliminated from the system by pre-electrolysis. Changing the Karl Fischer solution after each determination is not necessary because individual determinations can be carried out in succession in the same reagent solution. A requirement for this method is that each component of the test specimen is compatible with the other components, and no side reactions take place. Samples are usually transferred into the vessel as solutions by means of injection through a septum. Gases can be introduced into the cell by means of a suitable gas inlet tube. Precision in the method is predominantly governed by the extent to which atmospheric moisture is excluded from the system; thus, the introduction of solids into the cell may require precautions, such as working in a glove-box in an atmosphere of dry inert gas. Control of the system may be monitored by measuring the amount of baseline drift, which does not preclude the need of any blank correction when used as a vehicle for sample introduction. This method is particularly suited to chemically inert substances like hydrocarbons, alcohols, and ethers.

In comparison with the volumetric Karl Fischer titration, coulometry is a micro-method. When appropriate, the water may be desorbed or released from the sample by heat in an external oven connected with the vessel, to where it is transferred with the aid of an inert and dried gas such as pure nitrogen. Any drift due to the transport gas should be considered and corrected. Care should be taken in the selection of the heating conditions to avoid the formation of water coming from dehydration due to decomposition of the sample constituents, which may invalidate this approach.

**APPARATUS**

Any commercially available apparatus consisting of an absolutely tight system fitted with the necessary electrodes and a magnetic stirrer is appropriate. The instrument’s microprocessor controls the analytical procedure and displays the results. Calibration of the instrument is not necessary, as the current consumed can be measured absolutely.

**REAGENT**

See the manufacturer’s recommendations.

**TEST PREPARATION**

Where the specimen is a soluble solid, an appropriate quantity, accurately weighed, may be dissolved in anhydrous methanol or other suitable solvents.

Where the specimen is an insoluble solid, an appropriate quantity, accurately weighed, may be extracted using a suitable anhydrous solvent, and may be injected into the anolyte solution. Alternatively, an evaporation technique may be used in which water is released and evaporated by heating the specimen in a tube in a stream of dry inert gas. The gas is then passed into the cell.

Where the specimen is to be used directly without dissolving in a suitable anhydrous solvent, an appropriate quantity, accurately weighed, may be introduced into the chamber directly.

Where the specimen is a liquid, and is miscible with anhydrous methanol or other suitable solvents, an appropriate quantity, accurately weighed, may be added to anhydrous methanol or other suitable solvents.

**PROCEDURE**

Using a dry device, inject or add directly an accurately measured amount of the sample or sample preparation estimated to contain between 0.5 and 5 mg of water, or an amount recommended by the instrument manufacturer into the anolyte, mix, and perform the coulometric titration to the electrometric endpoint. Read the water content of the sample directly from the instrument’s display, and calculate the percentage that is present in the substance. Perform a blank determination, as needed, and make any necessary corrections.

**METHOD II (AZEOTROPIC—TOLUENE DISTILLATION)**

**Apparatus**

Use a 500-mL glass flask A connected by means of a trap B to a reflux condenser C by ground glass joints (see Figure 1).
The critical dimensions of the parts of the apparatus are as follows. The connecting tube D is 9–11 mm in internal diameter. The trap is 235–240 mm in length. The condenser, if of the straight-tube type, is approximately 400 mm in length and not less than 8 mm in bore diameter. The receiving tube E has a 5-mL capacity, and its cylindrical portion, 146–156 mm in length, is graduated in 0.1-mL subdivisions, so that the error of reading is not greater than 0.05 mL for any indicated volume. The source of heat is preferably an electric heater with rheostat control or an oil bath. The upper portion of the flask and the connecting tube may be insulated.

Clean the receiving tube and the condenser with a suitable cleanser, thoroughly rinse with water, and dry in an oven. Prepare the toluene to be used by first shaking with a small quantity of water, separating the excess water, and distilling the toluene.

**Procedure**

Place in the dry flask a quantity of the substance, weighed accurately to the nearest centigram, which is expected to yield 2–4 mL of water. If the substance is of a pasty character, weigh it in a boat of metal foil of a size that will just pass through the neck of the flask. If the substance is likely to cause bumping, add enough dry, washed sand to cover the bottom of the flask, or a number of capillary melting-point tubes, about 100 mm in length, sealed at the upper end. Place about 200 mL of toluene in the flask, connect the apparatus, and fill the receiving tube E with toluene poured through the top of the condenser. Heat the flask gently for 15 min and, when the toluene begins to boil, distill at the rate of about two drops per s until most of the water has passed over, then increase the rate of distillation to about four drops per s. When the water has apparently all distilled over, rinse the inside of the condenser tube with toluene while brushing down the tube with a tube brush attached to a copper wire and saturated with toluene. Continue the distillation for five min, then remove the heat, and allow the receiving tube to cool to room temperature. If any droplets of water adhere to the walls of the receiving tube, scrub them down with a brush consisting of a rubber band wrapped around a copper wire and wetted with toluene. When the water and toluene have separated completely, read the volume of water, and calculate the percentage that was present in the substance.

**METHOD III (GRAVIMETRIC)**

**Procedure for Chemicals**

Proceed as directed in the individual monograph preparing the chemical as directed under *Loss on Drying* (731).

**Procedure for Biologics**

Proceed as directed in the individual monograph.

**Procedure for Articles of Botanical Origin**

Place about 10 g of the drug, prepared as directed (see *Methods of Analysis* under *Articles of Botanical Origin* (561)) and accurately weighed, in a tared evaporating dish. Dry at 105° for 5 h, and weigh. Continue the drying and weighing at 1-h intervals until the difference between two successive weighings corresponds to not more than 0.25%.
CHARACTERIZATION OF CRYSTALLINE AND PARTIALLY CRYSTALLINE SOLIDS BY X-RAY POWDER DIFFRACTION (XRPD)

INTRODUCTION

Every crystalline phase of a given substance produces a characteristic X-ray diffraction pattern. Diffraction patterns can be obtained from a randomly oriented crystalline powder composed of crystallites or crystal fragments of finite size. Essentially three types of information can be derived from a powder diffraction pattern: the angular position of diffraction lines (depending on geometry and size of the unit cell), the intensities of diffraction lines (depending mainly on atom type and arrangement, and particle orientation within the sample), and diffraction line profiles (depending on instrumental resolution, crystallite size, strain, and specimen thickness).

Experiments giving angular positions and intensities of lines can be used for applications such as qualitative phase analysis (e.g., identification of crystalline phases) and quantitative phase analysis of crystalline materials. An estimate of the amorphous and crystalline fractions can also be made.

The X-ray powder diffraction (XRPD) method provides an advantage over other means of analysis in that it is usually nondestructive in nature (to ensure a randomly oriented sample, specimen preparation is usually limited to grinding). XRPD investigations can also be carried out under in situ conditions on specimens exposed to nonambient conditions such as low or high temperature and humidity.

PRINCIPLES

X-ray diffraction results from the interaction between X-rays and electron clouds of atoms. Depending on atomic arrangement, interferences arise from the scattered X-rays. These interferences are constructive when the path difference between two diffracted X-ray waves differs by an integral number of wavelengths. This selective condition is described by the Bragg equation, also called Bragg’s law (see Figure 1).

\[ 2d_{hkl} \sin \theta_{hkl} = n \lambda \]

Figure 1. Diffraction of X-rays by a crystal according to Bragg’s Law.

The wavelength, \( \lambda \), of the X-rays is of the same order of magnitude as the distance between successive crystal lattice planes, or \( d_{hkl} \) (also called d-spacings). \( \theta_{hkl} \) is the angle between the incident ray and the family of lattice planes, and \( \sin \theta_{hkl} \) is inversely proportional to the distance between successive crystal planes or d-spacings.

The direction and spacing of the planes with reference to the unit cell axes are defined by the Miller indices (hkl). These indices are the reciprocals, reduced to the next-lower integer, of the intercepts that a plane makes with the unit cell axes. The unit cell dimensions are given by the spacings \( a \), \( b \), and \( c \), and the angles between them \( \alpha \), \( \beta \), and \( \gamma \).

There are many other applications of the X-ray powder diffraction technique that can be applied to crystalline pharmaceutical substances, such as determination of crystal structures, refinement of crystal structures, determination of the crystallographic purity of crystalline phases, and characterization of crystallographic texture. These applications are not described in this chapter.
The interplanar spacing for a specified set of parallel hkl planes is denoted by \( d_{hkl} \). Each such family of planes may show higher orders of diffraction where the \( d \) values for the related families of planes \( nh, nk, nl \) are diminished by the factor \( 1/n \) (\( n \) being an integer: 2, 3, 4, etc.).

Every set of planes throughout a crystal has a corresponding Bragg diffraction angle, \( \theta_{hkl} \), associated with it (for a specific \( \lambda \)).

A powder specimen is assumed to be polycrystalline so that at any angle \( \theta_{hkl} \) there are always crystallites in an orientation allowing diffraction according to Bragg’s law.\(^2\) For a given X-ray wavelength, the positions of the diffraction peaks (also referred to as “lines”, “reflections”, or “Bragg reflections”) are characteristic of the crystal lattice (d-spacings), their theoretical intensities depend on the crystallographic unit cell content (nature and positions of atoms), and the line profiles depend on the perfection and extent of the crystal lattice. Under these conditions, the diffraction peak has a finite intensity arising from atomic arrangement, type of atoms, thermal motion, and structural imperfections, as well as from instrument characteristics.

The intensity is dependent upon many factors such as structure factor, temperature factor, crystallinity, polarization factor, multiplicity, and Lorentz factor.

The main characteristics of diffraction line profiles are position, peak height, peak area, and shape (characterized by, e.g., peak width, or asymmetry, analytical function, and empirical representation). An example of the type of powder patterns obtained for five different solid phases of a substance are shown in Figure 2.

![Figure 2. X-ray powder diffraction patterns collected for five different solid phases of a substance (the intensities are normalized).](image)

In addition to the diffraction peaks, an X-ray diffraction experiment also generates a more or less uniform background, upon which the peaks are superimposed. Besides specimen preparation, other factors contribute to the background—for example, sample holder, diffuse scattering from air and equipment, and other instrumental parameters such as detector noise and general radiation from the X-ray tube. The peak-to-background ratio can be increased by minimizing background and by choosing prolonged exposure times.

\(^2\) An ideal powder for diffraction experiments consists of a large number of small, randomly oriented spherical crystallites (coherently diffracting crystalline domains). If this number is sufficiently large, there are always enough crystallites in any diffracting orientation to give reproducible diffraction patterns.
INSTRUMENT

Instrument Setup

X-ray diffraction experiments are usually performed using powder diffractometers or powder cameras. A powder diffractometer generally comprises five main parts: an X-ray source; the incident beam optics, which may perform monochromatization, filtering, collimation, and/or focusing of the beam; a goniometer; the diffraction beam optics, which may include monochromatization, filtering, collimation, and focusing or parallelizing of beam; and a detector. Data collection and data processing systems are also required and are generally included in current diffraction measurement equipment.

Depending on the type of analysis to be performed (phase identification, quantitative analysis, lattice parameters determination, etc.), different XRPD instrument configurations and performance levels are required. The simplest instruments used to measure powder patterns are powder cameras. Replacement of photographic film as the detection method by photon detectors has led to the design of diffractometers in which the geometric arrangement of the optics is not truly focusing, but parafocusing, such as in Bragg-Brentano geometry. The Bragg-Brentano parafocusing configuration is currently the most widely used and is therefore briefly described here.

A given instrument may provide a horizontal or vertical θ/2θ geometry or a vertical θ/θ geometry. For both geometries, the incident X-ray beam forms an angle θ with the specimen surface plane, and the diffracted X-ray beam forms an angle 2θ with the direction of the incident X-ray beam (an angle θ with the specimen surface plane). The basic geometric arrangement is represented in Figure 3. The divergent beam of radiation from the X-ray tube (the so-called primary beam) passes through the parallel plate collimators and a divergence slit assembly and illuminates the flat surface of the specimen. All the rays diffracted by suitably oriented crystallites in the specimen at an angle 2θ converge to a line at the receiving slit. A second set of parallel plate collimators and a scatter slit may be placed either behind or before the receiving slit. The axes of the line focus and of the receiving slit are at equal distances from the axis of the goniometer. The X-ray quanta are counted by a radiation detector, usually a scintillation counter, a sealed-gas proportional counter, or a position-sensitive solid-state detector such as an imaging plate or CCD detector. The receiving slit assembly and the detector are coupled together and move tangentially to the focusing circle. For θ/2θ scans, the goniometer rotates the specimen around the same axis as that of the detector, but at half the rotational speed, in a θ/2θ motion. The surface of the specimen thus remains tangential to the focusing circle. The parallel plate collimator limits the axial divergence of the beam and hence partially controls the shape of the diffracted line profile.

A diffractometer may also be used in transmission mode. The advantage with this technology is to lessen the effects due to preferred orientation. A capillary of about 0.5- to 2-mm thickness can also be used for small sample amounts.

X-Ray Radiation

In the laboratory, X-rays are obtained by bombarding a metal anode with electrons emitted by the thermionic effect and accelerated in a strong electric field (using a high-voltage generator). Most of the kinetic energy of the electrons is converted to heat, which limits the power of the tubes and requires efficient anode cooling. A 20- to 30-fold increase in brilliance can be obtained by using rotating anodes and by using X-ray optics. Alternatively, X-ray photons may be produced in a large-scale facility (synchrotron).
The spectrum emitted by an X-ray tube operating at sufficient voltage consists of a continuous background of polychromatic radiation and additional characteristic radiation that depends on the type of anode. Only this characteristic radiation is used in X-ray diffraction experiments. The principal radiation sources used for X-ray diffraction are vacuum tubes using copper, molybdenum, iron, cobalt, or chromium as anodes; copper, molybdenum, or cobalt X-rays are employed most commonly for organic substances (the use of a cobalt anode can especially be preferred to separate distinct X-ray lines). The choice of radiation to be used depends on the absorption characteristics of the specimen and possible fluorescence by atoms present in the specimen. The wavelengths used in powder diffraction generally correspond to the Kα radiation from the anode. Consequently, it is advantageous to make the X-ray beam “monochromatic” by eliminating all the other components of the emission spectrum. This can be partly achieved using Kα filters—that is, metal filters selected as having an absorption edge between the Kα and Kβ wavelengths emitted by the tube. Such a filter is usually inserted between the X-ray tube and the specimen. Another more commonly used way to obtain a monochromatic X-ray beam is via a large monochromator crystal (usually referred to as a “monochromator”). This crystal is placed before or behind the specimen and diffracts the different characteristic peaks of the X-ray beam (i.e., Kα and Kβ) at different angles so that only one of them may be selected to enter into the detector. It is even possible to separate Kα1 and Kα2 wavelengths by using a specialized monochromator. Unfortunately, the gain in getting a monochromatic beam by using a filter or a monochromator is counteracted by a loss in intensity. Another way of separating Kα and Kβ wavelengths is by using curved X-ray mirrors that can simultaneously monochromate and focus or parallelize the X-ray beam.

RADIATION PROTECTION

Exposure of any part of the human body to X-rays can be injurious to health. It is therefore essential that whenever X-ray equipment is used, adequate precautions be taken to protect the operator and any other person in the vicinity. Recommended practice for radiation protection as well as limits for the levels of X-radiation exposure are those established by national legislation in each country. If there are no official regulations or recommendations in a country, the latest recommendations of the International Commission on Radiological Protection should be applied.

SPECIMEN PREPARATION AND MOUNTING

The preparation of the powdered material and the mounting of the specimen in a suitable holder are critical steps in many analytical methods, particularly for X-ray powder diffraction analysis, since they can greatly affect the quality of the data to be collected. The main sources of errors due to specimen preparation and mounting are briefly discussed in the following section for instruments in Bragg-Brentano parafocusing geometry.

Specimen Preparation

In general, the morphology of many crystalline particles tends to give a specimen that exhibits some degree of preferred orientation in the specimen holder. This is particularly evident for needle-like or plate-like crystals when size reduction yields finer needles or platelets. Preferred orientation in the specimen influences the intensities of various reflections so that some are more intense and others less intense, compared to what would be expected from a completely random specimen. Several techniques can be employed to improve randomness in the orientation of crystallites (and therefore to minimize preferred orientation), but further reduction of particle size is often the best and simplest approach. The optimum number of crystallites depends on the diffractometer geometry, the required resolution, and the specimen attenuation of the X-ray beam. In some cases, particle sizes as large as 50 µm will provide satisfactory results in phase identification. However, excessive milling (crystallite sizes less than approximately 0.5 µm) may cause line broadening and significant changes to the sample itself, such as

- specimen contamination by particles abraded from the milling instruments (mortar, pestle, balls, etc.),
- reduced degree of crystallinity,
- solid-state transition to another polymorph,
- chemical decomposition,
- introduction of internal stress, and
- solid-state reactions.

Therefore, it is advisable to compare the diffraction pattern of the nonground specimen with that corresponding to a specimen of smaller particle size (e.g., a milled specimen). If the X-ray powder diffraction pattern obtained is of adequate quality considering its intended use, then grinding may not be required.

It should be noted that if a sample contains more than one phase and if sieving is used to isolate particles to a specific size, the initial composition may be altered.

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3 Similarly, changes in the specimen can occur during data collection in the case of a nonequilibrium specimen (temperature, humidity).
Specimen Mounting

EFFECT OF SPECIMEN DISPLACEMENT

A specimen surface that is offset by D with reference to the diffractometer rotation axis causes systematic errors that are very difficult to avoid entirely; for the reflection mode, this results in absolute D · cosθ shifts in 2θ positions (typically of the order of 0.01° in 2θ at low angles

\[ \cos \theta = 1 \]

for a displacement D = 15 μm and asymmetric broadening of the profile toward low 2θ values. Use of an appropriate internal standard allows the detection and correction of this effect simultaneously with that arising from specimen transparency. This effect is by far the largest source of errors in data collected on well-aligned diffractometers.

EFFECT OF SPECIMEN THICKNESS AND TRANSPARENCY

When the XRPD method in reflection mode is applied, it is often preferable to work with specimens of “infinite thickness”. To minimize the transparency effect, it is advisable to use a nondiffracting substrate (zero background holder)—for example, a plate of single crystalline silicon cut parallel to the 510 lattice planes. One advantage of the transmission mode is that problems with sample height and specimen transparency are less important.

The use of an appropriate internal standard allows the detection and correction of this effect simultaneously with that arising from specimen displacement.

CONTROL OF THE INSTRUMENT PERFORMANCE

The goniometer and the corresponding incident and diffracted X-ray beam optics have many mechanical parts that need adjustment. The degree of alignment or misalignment directly influences the quality of the results of an XRPD investigation. Therefore, the different components of the diffractometer must be carefully adjusted (optical and mechanical systems, etc.) to adequately minimize systematic errors, while optimizing the intensities received by the detector. The search for maximum intensity and maximum resolution is always antagonistic when aligning a diffractometer. Hence, the best compromise must be sought while performing the alignment procedure. There are many different configurations, and each supplier’s equipment requires specific alignment procedures. The overall diffractometer performance must be tested and monitored periodically, using suitable certified reference materials. Depending on the type of analysis, other well-defined reference materials may also be employed, although the use of certified reference materials is preferred.

QUALITATIVE PHASE ANALYSIS (IDENTIFICATION OF PHASES)

The identification of the phase composition of an unknown sample by XRPD is usually based on the visual or computer-assisted comparison of a portion of its X-ray powder pattern to the experimental or calculated pattern of a reference material. Ideally, these reference patterns are collected on well-characterized single-phase specimens. This approach makes it possible in most cases to identify a crystalline substance by its d-spacings and by its relative intensities. The computer-aided comparison of the diffraction pattern of the unknown sample to the comparison data can be based on either a more or less extended 2θ range of the whole diffraction pattern or on a set of reduced data derived from the pattern. For example, the list of d-spacings and normalized intensities, \( I_{\text{norm}} \), a so-called (d, \( I_{\text{norm}} \)) list extracted from the pattern, is the crystallographic fingerprint of the material and can be compared to (d, \( I_{\text{norm}} \)) lists of single-phase samples compiled in databases.

For most organic crystals, when using Cu Kα radiation, it is appropriate to record the diffraction pattern in a 2θ-range from near 0° as possible to at least 40°. The agreement in the 2θ-diffraction angles between specimen and reference is within 0.2° for the same crystal form, while relative intensities between specimen and reference may vary considerably due to preferred orientation effects. By their very nature, variable hydrates and solvates are recognized to have varying unit cell dimensions, and as such, shifting occurs in peak positions of the measured XRPD patterns for these materials. In these unique materials, variance in 2θ positions of greater than 0.2° is not unexpected. As such, peak position variances such as 0.2° are not applicable to these materials. For other types of samples (e.g., inorganic salts), it may be necessary to extend the 2θ region scanned to well beyond 40°. It is generally sufficient to scan past the 10 strongest reflections identified in single-phase X-ray powder diffraction database files.

It is sometimes difficult or even impossible to identify phases in the following cases:

- noncrystallized or amorphous substances,
- the components to be identified are present in low mass fractions of the analyte amounts (generally less than 10% m/m),
- pronounced preferred orientation effects,
- the phase has not been filed in the database used,
- the formation of solid solutions,
- the presence of disordered structures that alter the unit cell,

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4 Note that a goniometer zero alignment shift would result in a constant shift on all observed 2θ-line positions; in other words, the whole diffraction pattern is, in this case, translated by an offset of \( Z \) in 2θ.

2 In the case of a thin specimen with low attenuation, accurate measurements of line positions can be made with focusing diffractometer configurations in either transmission or reflection geometry. Accurate measurements of line positions on specimens with low attenuation are preferably made using diffractometers with parallel beam optics. This helps to reduce the effects of specimen thickness.
the specimen comprises too many phases,
• the presence of lattice deformations,
• the structural similarity of different phases.

QUANTITATIVE PHASE ANALYSIS

If the sample under investigation is a mixture of two or more known phases, of which not more than one is amorphous, the percentage (by volume or by mass) of each crystalline phase and of the amorphous phase can in many cases be determined. Quantitative phase analysis can be based on the integrated intensities, on the peak heights of several individual diffraction lines,6 or on the full pattern. These integrated intensities, peak heights, or full-pattern data points are compared to the corresponding values of reference materials. These reference materials must be single phase or a mixture of known phases. The difficulties encountered during quantitative analysis are due to specimen preparation (the accuracy and precision of the results require, in particular, homogeneity of all phases and a suitable particle size distribution in each phase) and to matrix effects. In favorable cases, amounts of crystalline phases as small as 10% may be determined in solid matrices.

Polymorphic Samples

For a sample composed of two polymorphic phases \( a \) and \( b \), the following expression may be used to quantify the fraction \( F_\alpha \) of phase \( a \):

\[
F_\alpha = \frac{1}{1 + K(I_b/I_\alpha)}
\]

The fraction is derived by measuring the intensity ratio between the two phases, knowing the value of the constant \( K \). \( K \) is the ratio of the absolute intensities of the two pure polymorphic phases \( I_{oa}/I_{ob} \). Its value can be determined by measuring standard samples.

Methods Using a Standard

The most commonly used methods for quantitative analysis are
• the external standard method,
• the internal standard method, and
• the spiking method (also often called the standard addition method).

The external standard method is the most general method and consists of comparing the X-ray diffraction pattern of the mixture, or the respective line intensities, with those measured in a reference mixture or with the theoretical intensities of a structural model, if it is fully known.

To limit errors due to matrix effects, an internal reference material can be used that has a crystallite size and X-ray absorption coefficient comparable to those of the components of the sample and with a diffraction pattern that does not overlap at all that of the sample to be analyzed. A known quantity of this reference material is added to the sample to be analyzed and to each of the reference mixtures. Under these conditions, a linear relationship between line intensity and concentration exists. This application, called the internal standard method, requires precise measurement of diffraction intensities.

In the spiking method (or standard addition method), some of the pure phase \( a \) is added to the mixture containing the unknown concentration of \( a \). Multiple additions are made to prepare an intensity-versus-concentration plot in which the negative x-intercept is the concentration of the phase \( a \) in the original sample.

ESTIMATE OF THE AMORPHOUS AND CRYSTALLINE FRACTIONS

In a mixture of crystalline and amorphous phases, the crystalline and amorphous fractions can be estimated in several ways. The choice of the method used depends on the nature of the sample:
• If the sample consists of crystalline fractions and an amorphous fraction of different chemical compositions, the amounts of each of the individual crystalline phases may be estimated using appropriate standard substances, as described above. The amorphous fraction is then deduced indirectly by subtraction.
• If the sample consists of one amorphous and one crystalline fraction, either as a 1-phase or a 2-phase mixture, with the same elemental composition, the amount of the crystalline phase (the “degree of crystallinity”) can be estimated by measuring three areas of the diffractogram:

\[
A = \text{total area of the peaks arising from diffraction from the crystalline fraction of the sample,}
\]

\[
B = \text{total area below area A,}
\]

\[
C = \text{background area (due to air scattering, fluorescence, equipment, etc).}
\]

When these areas have been measured, the degree of crystallinity can be roughly estimated as:

\[
\% \text{ crystallinity} = \frac{100A}{(A + B - C)}
\]

6 If the crystal structures of all components are known, the Rietveld method can be used to quantify them with good accuracy. If the crystal structures of the components are not known, the Pawley method or the partial least-squares (PLS) method can be used.
It is noteworthy that this method does not yield an absolute degree of crystallinity values and hence is generally used for comparative purposes only. More sophisticated methods are also available, such as the Ruland method.

SINGLE CRYSTAL STRUCTURE

In general, the determination of crystal structures is performed from X-ray diffraction data obtained using single crystals. However, crystal structure analysis of organic crystals is a challenging task, since the lattice parameters are comparatively large, the symmetry is low, and the scattering properties are normally very low. For any given crystalline form of a substance, the knowledge of the crystal structure allows for calculating the corresponding XRPD pattern, thereby providing a preferred orientation-free reference XRPD pattern, which may be used for phase identification.
1. INTRODUCTION

This chapter provides information regarding acceptable practices for the use of analytical procedures to make decisions about pharmaceutical processes and products. Basic statistical approaches for decision making are described, and the comparison of analytical procedures is discussed in some detail.

NOTE—It should not be inferred that the analysis tools mentioned in this chapter form an exhaustive list. Other, equally valid, statistical methods may be used at the discretion of the manufacturer and other users of this chapter.

Assurance of the quality of pharmaceuticals is accomplished by combining a number of practices, including rigorous process and formulation design, validation, and development and execution of a robust control strategy. Each of these is dependent on reliable analytical procedures. In the development process, analytical procedures are utilized to ensure that the manufactured products are thoroughly characterized and to optimize the commercial manufacturing process. Final-product testing provides assurance that a product is consistently safe, efficacious, and in compliance with its specifications. Sound statistical approaches can be included in the commercial control strategy to further ensure that quality is preserved throughout the product lifecycle.

While not meant to be a complete account of statistical methodology, this chapter will rely upon some fundamental statistical paradigms. Key among these are population parameters, statistical design and sampling, and parameter uncertainty. Population parameters are the true but unknown values of a scientific characteristic of interest. While unknown, these can be estimated using statistical design and sampling. Statistical design is used to fully represent the population of interest and to manage the uncertainty of a result, while the random acquisition of test samples as well as their introduction into the measurement process helps to mitigate bias. Lastly uncertainty should be acknowledged between the true population parameter and the estimation process. Uncertainty can be expressed as either a probabilistic margin between the true and estimated value of a population parameter (e.g., a 95% confidence interval) or as the certainty that the population parameter is compliant with some expectation or acceptance criterion (predictive probability).

This chapter provides direction for scientifically acceptable administration of pharmaceutical studies using analytical data. Focus is on investigational studies where analytical data are generated from carefully planned and executed experiments, as well as confirmatory studies which are strictly regulated with limited flexibility in design and evaluation. This is in contrast to exploratory studies where historical data are utilized to identify trends or effects which are subject to further investigation. The quality of decisions made from either investigational or confirmatory studies is enhanced through adherence to the scientific method, and to the application of sound statistical principles. The steps of the scientific method can be summarized as follows.

Study objective. A pharmaceutical study can be as simple as testing and releasing a batch of commercial material or as complex as a comparison of analytical procedures. The same considerations apply to the simple study as they do to the complex study. Each study is associated with a population parameter which is used to address the study objective. For release the parameter might be the batch mean. For the analytical procedure comparison study, the parameter might be the difference in means produced by the analytical procedures. In each case an appropriate acceptance criterion on the population parameter is used to make a decision from the study.

Study design. The study should be designed with a structure and replication strategy which ensures representative consideration of the study objective, and which manages the risks associated with making an incorrect decision. Representative consideration of the study objective entails inclusion of samples and conditions which span the population being studied. Thus in release of a manufactured lot, samples across the range of manufacture might be included, while in a procedure comparison, each type and level of test sample might be considered. Similar consideration should be given to sample testing, where appropriate factors should be included in the procedure. The design should also acknowledge the study risks. The statistical basis for managing study risk is the reduction of the uncertainty in the estimation of the population parameter.

Study conduct. Once the study has been designed, samples are collected and data are generated using the analytical procedure. Effective use of randomization should be considered to minimize the impact of systematic variability or bias. Care should be taken during data collection to properly control the analytical procedure and to ensure accurate transcription and preservation of information. An adequate number of significant digits or decimal places should be saved and used throughout the calculations. Deviations from the study plan should be captured and assessed for their potential to impact study decisions.

Study analysis and decision. Prior to the final analysis, the data should be explored for data transformation and potential outliers. The analysis of the data should proceed according to the statistical methods considered during the study design. The analysis of the data and the reporting of study results should include proper consideration of uncertainty. Where appropriate, interval estimates should be used to communicate the robustness of the results (viz., the width of the interval) as well as facilitate communication of the study decision. A decision can be made when the objective of the study has been pre-formulated to make such a decision (e.g., as in an investigational or confirmatory study). The study may otherwise have been performed to estimate or describe some characteristic of a population. Caution should be taken in making decisions from post-hoc analyses of the data. This is called “data snooping” and can lead to inappropriate decisions.

This chapter has been written for the laboratory scientist and the statistician alike. The laboratory scientist is primarily skilled in the analytical procedures and the uses made of those procedures and should be aware of the value of statistical design and analysis in their practices. The statistician is primarily skilled in the design of empirical studies and the analysis which will return
reliable decisions and should appreciate the science and constraints within the laboratories. While variously knowledgeable in their understanding across specialties, both disciplines should value the essential components that comprise uses of analytical data.

More detailed discussion related to the steps of the scientific method will be given in Section 4, Study Considerations, and will be illustrated with an example in Section 5, Analytical Procedure Comparison. Prior to this Section 2 will review some Prerequisite Laboratory Practices and Principles, and Section 3 will describe and illustrate some Basic Statistical Principles and Uncertainty. A series of appendices is provided to illustrate topics related to the generation and use of analytical data. Control charts, equivalence and noninferiority testing, the principle of uncertainty, and Bayesian statistics are briefly discussed. The framework within which the results from a compendial test are interpreted is clearly outlined in General Notices, 7. Test Results. Selected references that might be helpful in obtaining additional information on the statistical tools discussed in this chapter are listed in Appendix 6: References at the end of the chapter. USP does not endorse these citations, and they do not represent an exhaustive list. Further information about many of the methods cited in this chapter may also be found in most statistical textbooks.

2. PREREQUISITE LABORATORY PRACTICES AND PRINCIPLES

The sound application of statistical principles to analytical data requires the assumption that such data have been collected in a traceable (i.e., documented) and unbiased manner. To ensure this, the following practices are beneficial.

**Sound Record Keeping**

Laboratory records are maintained with sufficient detail, so that other equally qualified analysts can reconstruct the experimental conditions and review the results obtained. When collecting data, the data should be obtained with more decimal places than the specification or study acceptance criterion requires. Rounding of results from uses of analytical data should occur only after final calculations are completed as per the General Notices. Study protocols and data analyses should be adequately documented so that a reviewer can understand the bases of the study design and the pathway to study decisions.

**Procedure Validation**

Analytical procedures used to release and monitor stability of clinical and commercial materials are appropriately validated as specified in Validation of Compendial Procedures (1225) or verified as noted in Verification of Compendial Procedures (1226). Further guidance is given in Statistical Tools for Procedure Validation (1210) and Biological Assay Validation (1033). Analytical procedures published in the USP–NF should be validated and meet the Current Good Manufacturing Practices (GMP) regulatory requirement for validation as established in the United States Code of Federal Regulations. When an analytical procedure is used in a non-GMP study, it’s good practice to ensure that the analytical procedure is adequately fit for use to support the study objective.

**Analytical Procedure and Sample Performance Verification**

Verifying an acceptable level of performance for an analytical procedure in routine or continuous use is a valuable practice. This may be accomplished by analyzing a control sample at appropriate intervals or locations, or using other means, such as, determining and monitoring variation among the standards, background signal-to-noise ratios, etc. This is commonly called system suitability. Attention to the measured performance attribute, such as charting the results obtained by testing of a control sample, can signal a change in performance that requires adjustment of the analytical system. Examples of control charts used to monitor analytical procedure performance are provided in Appendix 1: Control Charts.

Sample performance should also be verified during routine use of an analytical procedure. Variability among replicates as well as other sample specific performance attributes are used to ensure the reliability of sample measurement. A failure to meet a sample performance requirement can result in a retest of the sample after an appropriate investigation, versus a complete repeat of an analytical procedure run.

3. BASIC STATISTICAL PRINCIPLES AND UNCERTAINTY

This section introduces the concept of uncertainty, and couples this with familiar statistical tools which facilitate decisions made from analytical data. At the core of these principles and tools is an understanding of risk; more specifically the risks of making incorrect decisions based on analyses using measurement data. The consequences of these risks can be minor or significant, and should be factored into considerations related to both design of a study, and the interpretation of the results. The understanding of uncertainty is not new to the pharmaceutical industry, or more broadly throughout industries that make decisions from analytical data. The study of measurement and measurement uncertainty falls formally into the field of metrology (see Appendix 4: The Principle of Uncertainty). This section will frame the concept of uncertainty and illustrate some well-known statistical tools.

**Uncertainty**

A study is designed to reduce uncertainty in order to make more reliable decisions.
Uncertainty is associated with variability and communicates the closeness of a result to its true value. A fundamental aspect of uncertainty is probability which is sometimes expressed as confidence. The combination of the variability of the result from a study and confidence provides a powerful means to manage pharmaceutical decisions.

Uncertainty is directly related to risk. Risk may be expressed as a probability, but is more formally translated into cost, where cost is the opportunity loss due to making an incorrect decision times the probability of that loss. Here a loss may be quantifiable outcome such as the value of a lot of manufactured material, or less quantifiable such as the loss of patient benefit from a drug or biological.

Key to the concept of uncertainty is its relationship to the structure of variability. The overall variability of the result is a composition of many individual sources of variability. In a general sense one can manage the overall variability through refinement in one or some of those sources, or through strategic design (e.g., replication and blocking). In either case the effort results in higher certainty and lower risk.

## Basic Statistical Principles

All results from studies using analytical data are, at best, estimates of the true value because they contain uncertainty. Basic statistical principles related to estimation and uncertainty will be illustrated for the population mean of a manufactured lot.

### STATISTICAL MEASURES

Statistical measures used to estimate the center and dispersion of a population include the mean, standard deviation, and expressions derived there from, such as the percent coefficient of variation (%CV), sometimes referred to as percent relative standard deviation (%RSD). Such statistical measures can be used to calculate confidence intervals for summary parameters of the process generating the data, prediction intervals for capturing a single future measurement with specified confidence, or tolerance intervals capturing a specified proportion of the individual measurements with specified confidence.

### STATISTICAL ASSUMPTIONS

Statistical assumptions should be justified with respect to the underlying data generation process and verified using appropriate graphical or statistical tools. If one or more of these assumptions appear to be violated, alternative methods may be required in the evaluation of the data. In particular, most of the statistical measures and tests cited in this chapter rely on the assumptions that the underlying population of measurements is normally distributed and that the measurement results are independent and free from aberrant values or outliers. Assessment of the statistical assumptions and alternatives methods of analysis are discussed in Appendix 2: Models and Data Considerations.

### AVERAGING

A single analytical measurement may be useful in decision making if the sample has been prepared using a well-validated documented process, if the sample is representative of the population of interest, if the analytical errors are well known, and the measurement uncertainty associated with the single measurement is suitable to make the appropriate decision. The obtained analytical result may be qualified by including an estimate of the associated measurement uncertainty. For a single measurement this might come from the procedure validation or another source of prior knowledge.

There may be instances when one might consider averaging multiple measurements because the variability associated with the average value better meets the target measurement uncertainty requirement for its use. Thus, the choice of whether to use individual measurements or averages will depend upon the use of the measurement and the risks associated with making decisions from the measurement. For example, when multiple measurements are obtained on the same sample aliquot (e.g., from multiple injections of the sample in an HPLC procedure), it is generally advisable to average the individual values to represent the sample value. This should be supported by some routine suitability check on the variability amongst the individual measures. A decision rule, which defines and describes how a decision will be made, should be explicit to the population parameter of interest. When this is the center or the mean, then the average should be the basis of the rule. When this is variability amongst the individual measurements, then it should be the standard deviation, %CV, or range. Except in special cases (e.g., content uniformity), care should be taken in making decisions from individual measurements.

### ESTIMATING THE CENTER AND DISPERSION FROM A SAMPLE

Let \( Y_1, Y_2, \ldots, Y_n \) represent a sample of \( n \) observations from a population of interest. When the appropriate assumptions are met the most commonly used statistic to describe the center of the \( n \) observations is the sample or arithmetic mean \( \bar{Y} \):

\[
\bar{Y} = \frac{1}{n} \sum_{i=1}^{n} Y_i = \frac{Y_1 + Y_2 + \ldots + Y_n}{n} \quad (1)
\]

The dispersion can be estimated from the observations in various ways. The most common and useful assessment of the dispersion is the determination of the sample standard deviation. The sample standard deviation is calculated as.
The sample %CV is calculated as

\[
\% CV = \frac{S}{\bar{Y}} \times 100\% \quad (3)
\]

It should be noted that %CV is an appropriate measure of variability only if the property being measured is an absolute quantity such as mass. It is incorrect to report %CV for estimates reported as a percentage (e.g., percent purity) or which are in transformed units (e.g., pH or other logarithmic units; see Torbeck, 2010).

**Statistical Intervals**

Statistical intervals are used to describe or make decisions concerning population parameters or behavior of individual values. Three useful statistical intervals are prediction intervals, tolerance intervals, and confidence intervals. Prediction and tolerance intervals describe behavior of individual values and are discussed in (1210).

Confidence intervals are the basis for incorporating uncertainty into the estimate of a population parameter. A two-sided interval is composed of a lower bound \( LB \) and an upper bound \( UB \). For a confidence interval on a population parameter \( \theta \) these bounds are functions of the sample values such that

\[
Pr[LB \leq \theta \leq UB] = 100 \times (1 - \alpha)\% \quad (4)
\]

This leads to the construction of a \( 100 \times (1 - \alpha)\% \) two-sided confidence interval on a population mean

\[
LB = \bar{Y} - t_{1-\alpha/2:n-1} \cdot \frac{S}{\sqrt{n}}
\]
\[
UB = \bar{Y} - t_{1-\alpha/2:n-1} \cdot \frac{S}{\sqrt{n}} \quad (5)
\]

where \( n \) is the sample size and \( t_{1-\alpha/2:n-1} \) is the \( 1 - \alpha/2^{th} \) quantile of the cumulative Student \( t \) distribution having area \( 1 - \alpha/2 \) to the left and \( n - 1 \) degrees of freedom. One-sided intervals based on the individual bounds can be similarly defined.

The sampling and calculation process described above will provide a confidence interval that contains the true parametric value \( 100 \times (1 - \alpha)\% \) of the time. Alternatively one can utilize a Bayesian approach to derive an interval which contains, with probability \( 100 \times (1 - \alpha)\% \) the true value of the mean (12).

**4. Study Considerations**

There are a number of scientific and statistical considerations in conducting a study. These will be discussed in the context of the stages of the scientific method (see Introduction).

**Study Objective**

The study objective is a statement of the goal(s) of the study. Generally, the goals are placed into two categories: (1) estimation, and (2) inference. Estimation is the goal when the investigator wishes to report results that estimate true quantities that underlie the data generating process and are the subject of the study. In statistics these true quantities are called population parameters. Inference includes the additional step of using these estimates to make a decision about the unknown true value of the population parameter.

Numerical estimates can either be single numbers (point estimates), a range of numbers (interval estimates), or distributions (distributional estimates). A point estimate is a single number that “best” represents the unknown true value of a population parameter. The computed average or standard deviation of a data set sampled from the study population are examples of point estimates. “Best” in this context means the estimate is in some sense close to the unknown parameter value, although the difference between the estimate and the parameter will vary from sample to sample.

A point estimate reported alone has little utility because it doesn’t reflect the uncertainty manifested by the magnitude of the difference between the estimate and the true value. Statistical intervals can be used for this purpose. A discussion of statistical intervals can be found in Basic Statistical Principles and Uncertainty, Statistical Intervals. Interval estimates provide additional details that may be useful for risk based decision making.

Distributional estimates are used in Bayesian analysis to define expectations when the population parameter is viewed as a random variable. In particular, posterior distributions formed by combining prior and sample information are used to assign probabilities that the unknown parameter will fall in a given range. Appendix 5: Bayesian Inference describes the utility of distributional estimates in more detail.

A statistical paradigm used to express the objective of an inferential study is a statistical hypothesis test. A hypothesis test is expressed as a pair of statements called the null hypothesis \( H_0 \) and the alternative hypothesis \( (H_a) \). Both are expressed concerning some unknown population parameter. Population parameters are often denoted with Greek letters. The Greek letter theta \((\theta)\) will be used for illustration. A two-sided hypothesis test can be written as
where $\theta_0$ represents the hypothesized value for $\theta$. The alternative hypothesis is sometimes called the research hypothesis because it represents the objective of the study. As an example, consider the true slope of a linear model representing the average change in the purity of a compound over time. Traditionally, this parameter is represented with the Greek letter beta ($\beta$). An investigator intends to determine if there is evidence that the average change in purity is a function of time. That is, if it can be shown that the true value of the slope is non-zero. Accordingly, equation (6) is written as

$$H_0: \beta = 0$$
$$H_a: \beta \neq 0$$

It should be noted that this is called a two-sided hypothesis because the direction of the difference is unspecified. This would be the case if the study sought to determine either a positive change (increase in purity) or a negative change (decrease in purity). But this is unlikely to be the desired objective of the study. It’s more plausible that the study would strictly seek to determine if there is evidence that average purity decreases over time. This would be expressed as a one-sided hypothesis test as follows

$$H_0: \beta \geq 0$$
$$H_a: \beta < 0$$

The choice of two-sided or one-sided hypothesis test should be made when formulating the study objective, and prior to design and execution of the study. It should be based on a plausible scientific objective and should never be decided on the basis of the study results. Examples of two-sided and one-sided hypothesis tests will be given in Comparison of Analytical Procedures.

An additional consideration in formulating a study objective is the use of equivalence or noninferiority testing. These procedures require that the investigator formulate their hypotheses with a scientifically or practically meaningful objective. These will be illustrated in Comparison of Analytical Procedures and is discussed in detail in Appendix 3: Equivalence and Noninferiority Testing.

**Study Design**

Study design should ensure an acceptable level of uncertainty in an estimation study or an acceptable risk for drawing the wrong conclusion in a test of inference. This can be managed through use of statistical design tools, including blocking and replication. As discussed previously, the design should also consider strategic selection of samples and study conditions which are associated with experiences in normal practice.

**DESIGN OF AN ESTIMATION STUDY**

The design of an estimation study may use sufficient replication (sample size) and blocking to ensure desired control of the uncertainty in the result. To illustrate, consider estimation of a mean based on a simple random sample of $n$ units from a study population. The half width of the confidence interval (also called the margin of error) in equation (5) in Basic Statistical Principles and Uncertainty represents the uncertainty in the estimation of the mean. In planning the study, the margin of error can be defined to be no greater than a maximum allowable value $H$. Selecting the confidence level, $(1 - \alpha)$, and providing a preliminary estimate for the standard deviation ($S$), one can solve for a required sample size using the equation

$$n \geq \left( \frac{t_{1-\alpha/2} \cdot S}{H} \right)^2$$

Since the degrees of freedom of the $t$-value are a function of $n$, one must either solve equation (9) iteratively, or use an approximation by replacing the $t$-value with the associated $Z$-value. Preliminary estimates for $S$ are obtained from similar studies or through the advice of subject matter experts. Scale of the data (e.g., transformed or original scale) should be defined prior to obtaining the preliminary estimate of the standard deviation or defining $H$ (see Appendix 2: Models and Data Considerations for more on data transformation).

**DESIGN OF AN INFERENTIAL STUDY**

The design of an inferential study is based on controlling the risks of drawing the wrong conclusion. Following the paradigm of a hypothesis test, these risks are illustrated in Table 1 and Table 2.

<table>
<thead>
<tr>
<th>Table 1. Conclusions in a statistical test</th>
<th>If $H_0$ is true</th>
<th>If $H_0$ is false</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reject $H_0$</td>
<td>Wrong conclusion (Type I error)</td>
<td>Correct conclusion</td>
</tr>
</tbody>
</table>
Table 1. Conclusions in a statistical test (continued)

<table>
<thead>
<tr>
<th></th>
<th>If ( H_0 ) is true</th>
<th>If ( H_0 ) is false</th>
</tr>
</thead>
<tbody>
<tr>
<td>Do not reject ( H_0 )</td>
<td>Correct conclusion</td>
<td>Wrong conclusion (Type II error)</td>
</tr>
</tbody>
</table>

Table 2. Probabilities of a wrong conclusion

<table>
<thead>
<tr>
<th>Wrong Conclusion</th>
<th>Probability of Occurrence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I error</td>
<td>( \alpha ) (called the level of significance)</td>
</tr>
<tr>
<td>Type II error</td>
<td>( \beta ) (1 - ( \beta ) is called the power)</td>
</tr>
</tbody>
</table>

It is important to determine the required sample size to control the Type I error (\( \alpha \)) and Type II error (\( \beta \)) simultaneously. Formulas for sample sizes supporting an inferential study that depend on selected values of (\( \alpha \)) and (\( \beta \)) are available in many textbooks and software packages. These formulas become more complex when the design includes blocking or experimental factors such as analyst or day. Computer simulation is a useful tool in these more complex situations, and support of a statistician can be useful.

While replication is an effective strategy for reducing the impact of random variability on uncertainty and risk, blocking can be used to remove known sources of variability. For example, in a study to compare two analytical procedures, each procedure might be used to measure each sample unit of material. This results in the removal of the variability between sample units of material, which provides a reduced error term used to compare differences between the two procedures. By reducing the error term in this manner, the power of the experiment is increased for a fixed number of sample units. A numerical example is provided in Comparison of Analytical Procedures.

Study Conduct

It is important to avoid introducing systematic error or bias into the study results. Bias can be introduced through unintentional changes in experimental conditions, due to either known or unknown factors. Effective sampling and randomization are important considerations in mitigating the impact of bias. Sampling is performed after the study has been designed and constitutes the selection of test articles within the structure of the design. How to attain such a sample depends entirely on the question that is to be answered by the data. When possible, use of a random process is considered the most appropriate way of selecting samples.

The most straightforward type of random sampling is called simple random sampling. However, sometimes this method of selecting a random sample is not desirable because it cannot guarantee equal representation across study factors. The design of a study to release manufactured lots might incorporate factors such as selected times, locations, or parallel manufacturing streams (e.g., multiple filling lines). In this case a stratified sample whereby units are randomly selected from within each factor can be utilized. Regardless of the reason for taking a sample, a sampling plan should be established to provide details on how the sample is to be obtained to ensure that it is representative of the entirety of the population of interest.

Randomization should not be restricted to sampling. Study samples should be strategically entered into an analytical procedure using randomization, while blocking can be utilized to avoid confounding of the study objective with assay related factors.

Sometimes it’s impossible to utilize sampling plans which are random or systematic in nature. This is especially true when the population is infinite. In this case representativeness is addressed through study design including blocking, where factors which are known to be the key structural components of the population are used to represent the infinite population.

The optimal sampling and analytical testing strategy will depend on knowledge of the manufacturing, analytical measurement, and/or study related processes. In the case of sampling to measure a property of a manufactured lot, it is likely that the sampling will include some element of random selection. There should be sufficient samples collected for the original analysis, subsequent verification analyses, and other supporting analyses. In the case of sampling to address a more complex study, representativeness should be addressed through strategic design. It is recommended that the subject matter expert work with a statistician to help select the most appropriate sampling plan and design for the specified objective.

An additional consideration in the conduct of a study is data recording. Many institutions store data in a Laboratory Information Management System (LIMS). That data may be entered to the number of significant digits (decimals) of the reportable value for the test procedure. While this practice is appropriate for the purpose of reporting test data (such as in a Certificate of Analysis or in a regulatory dossier), it is inappropriate for data which may be used for subsequent analysis. This is noted in ASTM E29 where it is stated “As far as is practicable with the calculating device or form used, carry out calculations with the test data exactly and round only the final result”. Rounding intermediate calculated results contributes to the overall error in the final result. More on rounding is included in General Notices, 7.20 Rounding Rules and in Appendix 2: Models and Data Considerations.

Study Analysis

The culmination of a study is a statistical analysis of the data, and a decision in the case of an inferential study. Simple summaries such as group averages and appropriate measures of variability, as well as plots of the data and summary results facilitate the analysis and communication of the study results and decision. Summaries should be supplemented with confidence intervals or bounds, which express the uncertainty in the summary result (see Basic Statistical Principles and Uncertainty). Transformations based on either scientific information or empirical evidence can be considered, and screening for outlying values and subsequent investigations completed (see Appendix 2: Models and Data Considerations).
Many common statistical analysis tools are found in calculation programs such as spreadsheets and instrument software. Software which is dedicated to statistical analysis and modeling contain additional tools to evaluate assumptions associated with the analysis tools, such as normality, homogeneity of variance, and independence. Those with limited or no statistical training should consult a statistician throughout the process of conducting a study, including study design and analysis. Their statistical skills complement the laboratory skills in ensuring appropriate study design, analysis, and decisions.

The study considerations outlined in this section will be illustrated hereafter.

5. COMPARISON OF ANALYTICAL PROCEDURES

It is often necessary to compare two analytical procedures to determine if differences in accuracy and precision are less than an amount deemed practically important. For example, General Notices 6.30 describes the need to produce comparable results to the compendial method. Transfer of analytical procedures as described in Transfer of Analytical Procedures (1224) allows for comparative testing as an acceptable process. A change in a procedure includes a change in technology, a change in laboratory (called transfer), or a change in the reference standard in the procedure.

For purposes of this section, the terms old procedure and new procedure are used to represent a procedure before and after a change. Procedures with differences less than the practically important criterion are said to be equivalent or better (see Appendix 3: Equivalence and Noninferiority Testing). This section follows the outline described in Study Considerations highlighting the scientific method of (1) study objective, (2) study design, (3) study conduct, and (4) study analysis.

Study Objective of a Procedure Comparison

The study objective of a procedure comparison is to demonstrate that a new procedure performs equivalent to or better than an old procedure. There are two conceptual study populations: All future measurements made with the old procedure on a particular process, and all future measurements made with the new procedure on the same process. Each procedure is described in terms of the mean and standard deviation of the population of measurements. The mean and standard deviation of the reportable value of the new procedure are denoted by the Greek symbols \( \mu_N \) and \( \sigma_N \) respectively. The subscript \( N \) denotes the “new” procedure population. The mean and standard deviation of measurements using the “old” procedure are denoted \( \mu_O \) and \( \sigma_O \) respectively. These means and standard deviations are unknown, but conclusions concerning their potential equivalence or noninferiority (the new procedure is not inferior to the old procedure) are informed by estimates resulting from the experiment. Characteristics for comparison are most generally accuracy and precision across the range of the assay, and across conditions experienced during long term routine analysis. A risk analysis should be performed to identify such conditions. Discussion of accuracy and precision are found in (1225).

ACCURACY

To compare accuracy of two procedures, one compares the procedure means. In particular, accuracy is compared using the absolute value of the true difference in means,

\[
|\mu_O - \mu_N| = |\mu_N - \mu_O|. \quad (10)
\]

The objective of such a study is to demonstrate that \( |\mu_O - \mu_N| \) is less than a value deemed to be practically important, \( d \). As an example, \( d \) may represent a numerical value that is small enough so that an increase in bias of this magnitude does not negatively impact decisions concerning lot disposition (i.e., conformance to specifications). The hypotheses used in an equivalence test are

\[
\begin{align*}
H_C: |\mu_O - \mu_N| &\geq d \\
H_E: |\mu_O - \mu_N| &< d
\end{align*} \quad (11)
\]

(see Appendix 3: Equivalence and Noninferiority Testing).

Probably the most difficult aspect of conducting an equivalence test is determination of \( d \). Typically, \( d \) is determined in partnership between the analytical chemist and the statistician based on combined manufacturing and scientific knowledge. Definitions of \( d \) vary across companies based on differing risk profiles and experience. In some cases there exists a large amount of legacy data that may inform the decision, while in other cases there may be only limited data. An example where \( d \) is based on requirements of a manufacturing process follows in the section Determination of \( d \) and \( k \).

PRECISION

To compare precision of two procedures, one compares the procedure standard deviations. Whereas a comparison of means involves a difference, a comparison of standard deviations involves the ratio

\[
\frac{\sigma_N}{\sigma_O} \quad (12)
\]

The study objective is to demonstrate that the ratio in equation (12) is less than a practically important value \( k \). The noninferiority hypotheses are
\[
\frac{\sigma_N}{\sigma_O} \geq k \\
\frac{\sigma_N}{\sigma_O} < k
\] (13)

(see Appendix 3: Equivalence and Noninferiority Testing). The selection of \( k \) should be in alignment with the selection of \( d \) for the accuracy assessment. This process is demonstrated in the following section.

DETERMINATION OF \( d \) AND \( k \)

Values of \( d \) and \( k \) for the tests of accuracy and precision should be internally consistent. To demonstrate, consider a case where historical measurements using an old procedure for a monitored process have a process mean of \( \mu_O = 100 \) units and a combined process and analytical variance of \( \sigma^2_L + \sigma^2_O = 0.80 \) where \( \sigma^2_L \) represents lot-to-lot variability of the manufacturing process. Historic measurements of a reference standard provide the estimate \( \sigma^2_O = 0.16 \) so that the assumed value of the lot variance is \( \sigma^2_L = 0.80 - 0.16 = 0.64 \). The process specifications are the lower specification limit \( LSL = 96 \) units and the upper specification limit \( USL = 104 \) units. The same manufacturing process measured with the new procedure can be represented as having mean \( \mu_N = \mu_O + d \) and total process and analytical variance \( \sigma^2_L + \sigma^2_N = \sigma^2_L + k^2 \sigma^2_O \).

Kringle et al. (2001) recommend selecting values of \( d \) and \( k \) consistent with a rule that states the proportion of product that falls outside of specification (OOS) when measured with the new procedure is acceptable. Table 3 reports the OOS rate when the process is in control and measured with the new procedure for several values of \( d \) and \( k \). (Since the specifications are symmetric around \( \mu_O \), negative values of \( d \) provide the same OOS rates as the positive values shown in the Table 3).

Table 3. OOS rate with new procedure for values of \( d \) and \( k \)

<table>
<thead>
<tr>
<th>( d )</th>
<th>( k=1 )</th>
<th>( k=1.5 )</th>
<th>( k=2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.001%</td>
<td>0.01%</td>
<td>0.04%</td>
</tr>
<tr>
<td>1</td>
<td>0.04%</td>
<td>0.14%</td>
<td>0.40%</td>
</tr>
<tr>
<td>2</td>
<td>1.27%</td>
<td>2.28%</td>
<td>3.85%</td>
</tr>
</tbody>
</table>

Table 3 assumes the process is normal and the probability in any cell is given by the equation

\[
Pr(OOS) = 1 - Pr(96 \leq \text{Sampled process value} \leq 104) = 1 - \Phi \left( \frac{104 - (100 + d)}{\sqrt{0.64 + k^2 \times 0.16}} \right) - \Phi \left( \frac{96 - (100 + d)}{\sqrt{0.64 + k^2 \times 0.16}} \right)
\] (14)

where \( \Phi(\cdot) \) represents the cumulative probability function of the standard normal distribution. Suppose that the risk profile allows an OOS rate no greater than 1.0\%. Based on Table 3, a consistent set of criteria are \( d=1 \) and \( k=2 \).

Study Design of a Procedure Comparison

The study design for comparing the old and new analytical procedures is comprised of the selection of test materials, experimental design, and sample size determination (the so-called power calculation). Results for two scenarios are provided in this section. The first scenario considers samples from homogeneous test material, and the second scenario considers test material with variation across sample units.

SCENARIO 1: HOMOGENEOUS TEST MATERIAL

In this scenario, test samples of homogeneous material are selected and measured using one of the procedures on each test sample. There are \( n_o \) samples measured with the old procedure and \( n_N \) samples measured with the new procedure. It is recommended to design the study so that \( n_o = n_N \). Table 4 presents this design which is referred to as an independent two-sample design.

Table 4. Independent two-sample design

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>New Procedure</th>
<th>Old Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>( y_{nN} )</td>
<td>( y_{nO} )</td>
</tr>
<tr>
<td>2</td>
<td>( y_{nN} )</td>
<td>( y_{nO} )</td>
</tr>
<tr>
<td>( \bar{y} )</td>
<td>( \bar{y}_{nN} )</td>
<td>( \bar{y}_{nO} )</td>
</tr>
</tbody>
</table>
Table 4. Independent two-sample design (continued)

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>New Procedure</th>
<th>Old Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>( n_N + 1 )</td>
<td>( \bar{Y}_N )</td>
<td>( \bar{Y}_O )</td>
</tr>
<tr>
<td>( n_N + 2 )</td>
<td>( \frac{\sum Y_{Nj}}{n_N} )</td>
<td>( \frac{\sum Y_{Oj}}{n_O} )</td>
</tr>
<tr>
<td>( n_N + n_o )</td>
<td>( \sum (Y_{Nj} - \bar{Y}_N)^2 )</td>
<td>( \sum (Y_{Oj} - \bar{Y}_O)^2 )</td>
</tr>
</tbody>
</table>

Sample Mean

\[
\text{Sample Mean} = \frac{\sum_{j=1}^{n_N} Y_{Nj}}{n_N} = \frac{\sum_{j=1}^{n_O} Y_{Oj}}{n_O}
\]

Sample Variance

\[
\text{Sample Variance} = \frac{\sum_{j=1}^{n_N} (Y_{Nj} - \bar{Y}_N)^2}{n_N - 1} = \frac{\sum_{j=1}^{n_O} (Y_{Oj} - \bar{Y}_O)^2}{n_O - 1}
\]

For the comparison of means the estimator of interest is the difference of sample means, \( \bar{Y}_N - \bar{Y}_O \) which has variance

\[
\text{Var}(\bar{Y}_N - \bar{Y}_O) = \frac{\sigma_N^2}{n_N} + \frac{\sigma_O^2}{n_O}
\]

(15)

Power calculations are needed to ensure the sample size is great enough to find evidence that \( H_0 \) is true when such is the case. For testing the equivalence hypotheses in equation (11) assuming \( \sigma_N = \sigma_O \), Bristol (1993) recommends the sample size formula

\[
n_N = n_O = 2 \times \frac{\left( \frac{Z_{1-\alpha} + Z_{1-\beta}}{d - \mu_D} \right) \times \sigma_O}{\mu_D} + 1
\]

(16)

where \( Z_{1-\alpha} \) and \( Z_{1-\beta} \) are standard normal percentiles with area 1\( - \alpha \) and 1\( - \beta \) respectively, to the left. The Type I error rate is \( \alpha \) and the Type II error rate is \( \beta \). To make this calculation consistent with the case where \( \sigma_N \) can be as great as \( k \sigma_O \), a recommended modification is

\[
n_N = n_O = (1 + k)^2 \times \frac{\left( \frac{Z_{1-\alpha} + Z_{1-\beta}}{d - \mu_D} \right) \times \sigma_O}{\mu_D} + 1
\]

(17)

The information provided earlier to select \( d=1 \) and \( k=2 \) is now used to determine sample size for the study. For the test of equivalence of means, it is desired to have a high probability of passing when the two means are equal, that is when \( \mu_D = 0 \). So setting \( \beta = 0.10 \) and \( \alpha = 0.05 \) with \( \sigma_O = \sqrt{0.16} = 0.4 \), the required sample size for both the new and old procedures using equation (17) is

\[
n_N = n_O = (1 + 2) \times \frac{(1.645 + 1.282) \times 0.4}{1 - 0} + 1 = 7.9
\]

(18)

which is rounded up to 8 for each procedure (for 16 total test samples).

To test the noninferiority hypotheses in equation (13), it is desired to have a high power when \( \sigma_N = \sigma_O \), The required sample size is obtained by solving for \( n_N \) and \( n_O \) iteratively using the equation

\[
1 - \beta = \Pr \left( \frac{\sigma^2 k^2}{\sigma_N^2} \times F_{\alpha; n_N - 1, n_O - 1} \right)
\]

(19)

where \( F \) is a random variable following the \( F \)-distribution with degrees of freedom \( n_N - 1 \) and \( n_O - 1 \). As noted earlier, it is recommended that \( n_N = n_O \) and the sample size is the greater of the requirements from equations (17) and (19). Table 5 reports the power for sample size combinations using previous information when \( \alpha = 0.05 \) and \( \sigma_N = \sigma_O = 0.4 \).
Table 5. Power calculation for noninferiority test with $\alpha = 0.05$

<table>
<thead>
<tr>
<th>$n_N$</th>
<th>$n_O$</th>
<th>$\frac{\sigma^2_k}{\sigma^2_N}$</th>
<th>$F: n_N - 1, n_O - 1$</th>
<th>$\frac{\sigma^2_k^2}{\sigma^2_N}$</th>
<th>$F: n_N - 1, n_O - 1$</th>
<th>Power when $\sigma_N = \sigma_O$</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>8</td>
<td>4</td>
<td>0.264</td>
<td>1.056</td>
<td>0.528</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>14</td>
<td>4</td>
<td>0.388</td>
<td>1.552</td>
<td>0.781</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>15</td>
<td>4</td>
<td>0.403</td>
<td>1.610</td>
<td>0.808</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>19</td>
<td>4</td>
<td>0.451</td>
<td>1.804</td>
<td>0.890</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>20</td>
<td>4</td>
<td>0.461</td>
<td>1.845</td>
<td>0.904</td>
<td></td>
</tr>
</tbody>
</table>

From Table 5 it is seen that the sample of size 8 required for the test of equivalence of means does not provide acceptable power for the noninferiority test (Power = 0.528). This is because estimates of standard deviations have greater uncertainty than estimates of means. Practicality often dictates that one select a greater value for $\beta$ in a test of noninferiority than in a test for equivalence of means. In the present example, $\beta$ is selected as 0.20 and a sample size of 15 per procedure (30 test samples in total) is selected for the design.

When a comparison is made between laboratories (as during procedure transfer) it’s important to keep in mind that in order to be representative of future testing, the study design should include factors which have significant impact on the long term performance of the procedure. As noted previously, this may include analyst, but may also require that multiple instruments and batches of key reagents be included in the design. These may be nested or crossed. Failing to do so may underestimate the variability or confound the effects of some factors with the difference between labs. In general factors such as analysts where levels are unique within each laboratory might be nested within each lab, while factors such as reagent lots which might be routinely shared across laboratories could be crossed with laboratory. As such, the estimates of variability used in these equations should be representative of the variability induced by these factors. The best estimates of variability come from data collected on samples tested across a broad period of time, such as stability samples and an assay control. More considerations of this nature are described in (1210).

SCENARIO 2: VARIATION ACROSS TEST SAMPLES

It is often desirable to compare procedures across manufactured lots or use different manufactured levels of an analyte. This is important if the study objective is to ensure the range of the procedure in the new laboratory, or when the procedure is intended to measure degraded samples. This selection of test material introduces a new source of variation to Scenario 1 that must be considered during the study design in order to most efficiently compare the two procedures.

The recommended design in Scenario 2 is a paired design in which each test sample is measured independently by both procedures, instead of having each test sample randomly measured by only one procedure as in Scenario 1. The term “Test Sample” is referred to as a blocking factor because observations within the same block are differenced (see Study Considerations). This has the effect of removing the variation across test samples from the analysis. Table 6 presents a schematic illustration of the paired design using $n$ test samples.

Table 6. Paired design

<table>
<thead>
<tr>
<th>Test Sample</th>
<th>New Procedure</th>
<th>Old Procedure</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$Y_{N1}$</td>
<td>$Y_{O1}$</td>
<td>$D_1 = Y_{N1} - Y_{O1}$</td>
</tr>
<tr>
<td>2</td>
<td>$Y_{N2}$</td>
<td>$Y_{O2}$</td>
<td>$D_2 = Y_{N2} - Y_{O2}$</td>
</tr>
<tr>
<td>⋮</td>
<td>⋮</td>
<td>⋮</td>
<td>⋮</td>
</tr>
<tr>
<td>$n$</td>
<td>$Y_{Nn}$</td>
<td>$Y_{On}$</td>
<td>$D_n = Y_{Nn} - Y_{On}$</td>
</tr>
</tbody>
</table>

Sample Mean

| $\overline{Y}_N$ | $\overline{Y}_O$ |

Sample Variance

| NA | NA |

$\overline{D} = \frac{\sum_{i=1}^{n} D_i}{n}$

$\sigma^2_D = \frac{\sum_{i=1}^{n} (D_i - \overline{D})^2}{n - 1}$
Using the paired design with \( n \) lots, the variance of \( \bar{D} \) is \( \sigma_N^2 + \sigma_O^2)/n \) because the variability due to lots disappears when results on the same lot are differenced. The unbiased estimator of \( \sigma_N^2 + \sigma_O^2 \) is \( s_D^2 \).

The sample size formula for satisfying the mean test requirements for a paired design adjusting for the fact that \( \sigma_N^2 \) can be as great as \( k^2 \sigma_O^2 \) is

\[
\frac{Z_1 - \alpha + Z_1 - \beta}{d - \mu_D} \times \sqrt{\sigma_N^2 + \sigma_O^2} + 1
\]

\[
= (1 + k^2) \times \left( \frac{Z_1 - \alpha + Z_1 - \beta}{d - \mu_D} \times \sigma_O \right)^2 + 1
\]

which is the same formula shown in equation (17).

Using the same planning data from Scenario 1, the test for equivalence of means with \( \beta = 0.10 \) when \( \mu_D = 0 \) and \( \alpha = 0.05 \) is as before

\[
\frac{Z_1 - \alpha + Z_1 - \beta}{d - \mu_D} \times \sqrt{\sigma_N^2 + \sigma_O^2} + 1
\]

which is rounded up to 8 test samples (which are each measured once by each procedure). When using a paired design for the test of non-inferiority, the ability to find a good estimate of \( \sigma_N^2 \) is critical. Good estimates of \( \sigma_O^2 \) are often available from previous method validation studies or repeated measurements of an assay control. If no such estimate exists, it is necessary to modify the design in Table 6 and record two independent measurements with each procedure on each test sample. Independent estimates of both \( \sigma_N^2 \) and \( \sigma_O^2 \) can then be computed from the differences of the two paired values as shown in the section Study Analysis of a Procedure Comparison that follows.

If a good estimate for \( \sigma_O^2 \) is available, the required sample size for the noninferiority test is derived iteratively from the equation

\[
1 - \beta = Pr[W < \left( \frac{k^2 + 1}{\sigma_N^2 + \sigma_O^2} \right) \times \chi^2_{\alpha;n-1}] \]

where \( W \) is a chi-squared random variable with \( n - 1 \) degrees of freedom. Table 7 reports the power for sample size combinations when \( \alpha = 0.05 \) and \( \sigma_N = \sigma_O = 0.4 \).

<table>
<thead>
<tr>
<th>( n )</th>
<th>( \frac{(k^2 + 1)^2}{\sigma_N^2 + \sigma_O^2} )</th>
<th>( \chi^2_{\alpha;n-1} )</th>
<th>Power when ( \sigma_N = \sigma_O )</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>2.5</td>
<td>2.167</td>
<td>5.418</td>
</tr>
<tr>
<td>17</td>
<td>2.5</td>
<td>7.962</td>
<td>19.904</td>
</tr>
<tr>
<td>18</td>
<td>2.5</td>
<td>8.672</td>
<td>21.679</td>
</tr>
<tr>
<td>22</td>
<td>2.5</td>
<td>11.591</td>
<td>28.978</td>
</tr>
<tr>
<td>23</td>
<td>2.5</td>
<td>12.338</td>
<td>30.845</td>
</tr>
</tbody>
</table>

To obtain a power of 0.80 when the two standard deviations are equal, a sample of 18 test samples is required. Note that each test sample need not be unique. For example, if samples are being selected from three lots of product, one could select six test samples from each lot.

**Study Conduct of a Procedure Comparison**

When conducting the study, it is important to observe the random assignment of test samples to procedures in Scenario 1 in order to guard against possible bias. If repeated measurements are used in Scenario 2 to provide individual estimates of \( \sigma_O^2 \)
and \( \sigma_N^2 \), then independent measurements are needed. This will require independent preparations for each portion of the test sample.

**Study Analysis of a Procedure Comparison**

Two examples are provided to demonstrate the described formulas. Data in the examples were simulated from a population where \( \mu_N = \mu_O = 100 \) and \( \sigma_N^2 = \sigma_O^2 \). These values were selected to demonstrate the computed sample sizes are sufficient under the assumed conditions.

**SCENARIO 1: HOMOGENEOUS TEST MATERIAL**

Table 8 reports a sample data set with \( n_N = n_O = 15 \).

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Sample Mean</th>
<th>Sample Variance</th>
</tr>
</thead>
<tbody>
<tr>
<td>New</td>
<td>100.08</td>
<td>0.214</td>
</tr>
<tr>
<td>Old</td>
<td>99.85</td>
<td>0.159</td>
</tr>
</tbody>
</table>

Accuracy is tested using the hypotheses in equation (11) by constructing a 100(1 − 2\( \alpha \))% confidence interval on \( \mu_o \) using the equation

\[
\bar{Y}_N - \bar{Y}_O \pm t_{1-\alpha;df}\sqrt{\frac{s_N^2}{n_N} + \frac{s_O^2}{n_O}}
\]

\[
df = \frac{s_N^2 + s_O^2}{\frac{n^2}{n_N(n_N-1)} + \frac{n^2}{n_O(n_O-1)}}
\]

where \( t_{1-\alpha;df} \) is a quantile from a central t-distribution with area \( 1 - \alpha \) to the left and degrees of freedom \( df \). The null hypothesis in equation (11) is rejected, and equivalence demonstrated if the entire confidence interval computed from equation (23) falls in the range from \(-d\) to +\(d\). This is the TOST described in Appendix 3: Equivalence and Noninferiority Testing and has a Type I error rate of \( \alpha \). With some software packages such as Excel, non-integer \( df \) values are not accepted when determining the t-value. In this case, simply round to the nearest integer.

The 90% two-sided confidence interval that provides a Type I error rate of 0.05 computed from equation (23) is

\[
\bar{Y}_N - \bar{Y}_O \pm 1.703 \sqrt{\frac{0.214^2}{15} + \frac{0.159^2}{15} - \frac{0.04}{15}}
\]

Since the computed confidence interval falls entirely in the range between \(-1\) and \(+1\) (i.e., \(-d\) to +\(d\)) equivalence of means has been demonstrated.

Precision is tested using the hypotheses in equation (13) by constructing a 100(1 − \( \alpha \))% one-sided upper confidence bound on the ratio \( \sigma_N/\sigma_O \) using the formula

\[
\frac{S_N}{S_O} \leq \frac{F_{\alpha; n_N-1, n_O-1}}{n_N-1, n_O-1}
\]
where \( F_{\alpha, n_N - 1, n_O - 1} \) is the \( F \)-quantile with area \( \alpha \) to the left and degrees of freedom \( n_N - 1 \) and \( n_O \). If the upper bound computed with equation (25) is less than \( k \), the null hypothesis is rejected and one concludes noninferiority of the standard deviation of the new procedure. This test has a Type I error rate of \( \alpha \).

The 95\% upper bound on \( \sigma_N/\sigma_O \) computed from equation (25) is

\[
U = \frac{0.214}{0.195} \sqrt{\frac{0.350}{0.402}} = 1.83
\]

Since this upper bound is less than \( k = 2 \), noninferiority of the standard deviation of the new procedure has been demonstrated.

**SCENARIO 2: VARIATION ACROSS TEST SAMPLES**

Table 9 provides summary results for 18 test samples in a paired design with \( \bar{D} = \bar{Y}_N - \bar{Y}_O \).

**Table 9. Data from simulated paired design with \( n=18 \)**

<table>
<thead>
<tr>
<th>Sample Mean</th>
<th>Sample Variance</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \bar{D} = 0.39 )</td>
<td>( \frac{s_D^2}{n} = 0.350 )</td>
</tr>
</tbody>
</table>

The 90\% confidence interval on the difference in means for a paired design used to test equivalence of means with the data from Table 9 is

\[
S_{\bar{D}} \pm t_{0.95; n-1} \sqrt{\frac{s_D^2}{n}} = 0.39 \pm 1.74 \sqrt{0.350} / 18 = 0.15 \text{ to } 0.63
\]

Since the computed confidence interval falls entirely in the range between \( -1 \) and \( +1 \) equivalence of means has been demonstrated.

The noninferiority hypotheses in equation (13) can be tested by constructing a 100(1 - \( \alpha \))\% upper confidence bound on \( \sigma_N/\sigma_O \) using the formula

\[
\frac{\chi^2_{\alpha, n-1}}{\sigma_O^2} \leq \frac{(n-1)s_D^2}{2} - 1
\]

where \( \chi^2_{\alpha, n-1} \) is a percentile from the chi-squared distribution with area \( \alpha \) to the left and degrees of freedom \( n - 1 \). If this upper bound is less than \( k \), the null hypothesis is rejected and noninferiority has been demonstrated.

From historical data used to plan the sample size, a good estimate of the old procedure variance is \( \sigma_O^2 = 0.16 \). Using the confidence bound in equation (28), the 95\% upper confidence bound on \( \sigma_N/\sigma_O \) is

\[
U = \frac{(n-1)s_D^2}{0.16 \times 8.67} = \frac{18 - 1}{0.350 - 1} = 1.81
\]

Since this upper bound is less than \( k=2 \), noninferiority of the standard deviation of the new procedure has been demonstrated.

If a good estimate of \( \sigma_O^2 \) is not available, the design requires replicate measures for each procedure on each test sample.

Independent estimates of the analytical variances are computed using the formulas

\[
\begin{align*}
S_{DN}^2 &= \frac{n}{n-1} \left( \frac{Y_{i1} - Y_{i2} - \bar{D}_N}{\sqrt{2}} \right)^2 \\
S_{DO}^2 &= \frac{n}{n-1} \left( \frac{Y_{i1} - Y_{i2} - \bar{D}_O}{\sqrt{2}} \right)^2
\end{align*}
\]
where $Y_{jN}$ is the first measurement on test sample $j$ with method $N$, $Y_{jO}$ is the second measurement on test sample $j$ with method $N$, $Y_{j1}$ is the first measurement on test sample $j$ with method $O$, and $Y_{j2}$ is the second measurement on test sample $j$ with method $O$. The resulting $100(1 - \alpha)\%$ one-sided upper confidence bound on the ratio $\sigma_j/\sigma_O$ is

$$
\frac{S_{DN}}{S_{DO}} \leq \frac{1}{\sqrt{n-1}} \text{F}_{\alpha, n-1, n-1} (31)
$$

where $F_{\alpha, n-1, n-1}$ is the $F$-quantile with area $\alpha$ to the left and degrees of freedom $n - 1$ and $n - 1$, and $n$ is the number of test samples (each with four independent measures). If this formulation is needed, then define $D_i = (Y_{jN} + Y_{jO}) - (Y_{j1} + Y_{j2})/\sqrt{2}$ in the test for mean equivalence.

**APPENDIX 1: CONTROL CHARTS**

Control charts are used in the pharmaceutical industry to monitor the performance of manufacturing processes and analytical procedures. Using the vernacular of the scientific method, control charts are a tool to study these process populations, requiring a carefully developed objective, a strategic design, plans for implementation, and appropriate analysis. This appendix will discuss and illustrate the design and analysis of various control chart tools, as well as provide rules which are commonly used to make decisions.

Through its lifecycle a process or a procedure can be influenced by known changes or unforeseen variability. For a manufacturing process this might impact the quality of the product or indicate the need to take action. For an analytical procedure which is routinely used to aid decision-making, this might increase the risk of drawing the wrong conclusion from a study or likewise indicate the need for action. Thus, it is important to continuously verify performance and provide ongoing assurance of a state of control. To this end, data from a manufacturing process or that relate to procedure performance are collected and analyzed. For a manufacturing process these may include process parameters and test results on manufactured materials. For an analytical procedure they can include analytical results for controls, standards used during the analysis, and system suitability data. It’s important to note that the control samples are used to monitor the performance of the procedure and are not an indicator of the product performance or characteristics (FDA ISO 17025). For purposes of this appendix the term process will be used to refer to both a manufacturing process and an analytical procedure.

Although various trending methods exist, control charts are one of the most simple and effective graphical tools for such analysis. There are many types of control charts including the following:

- Individual (I) chart for plotting individual values over time,
- X-bar chart for plotting sample means over time,
- Range (R) chart for plotting sample ranges over time,
- Moving range (MR) chart for plotting moving ranges over time,
- S-chart for plotting sample standard deviations over time, and
- Exponentially weighted moving average (EWMA) and cumulative sum (CUSUM) charts which are used when small shifts in the mean of the procedure are of interest.

A typical control chart consists of a centerline and lower and upper control limits. The centerline represents the center of the distribution of a variable measured in the process. The two control limits are determined such that if the process performs as intended, nearly all results will fall within the two limits. Observations outside the limits or points within the limits that indicate a systematic or non-random pattern are indicative of a potential performance issue. Non-systematic patterns have been defined requiring a carefully developed objective, a strategic design, plans for implementation, and appropriate analysis. This appendix procedures. Using the vernacular of the scientific method, control charts are a tool to study these process populations, indicating the need for action. Thus, it is important to continuously verify performance and provide ongoing assurance of a state of control. To this end, data from a manufacturing process or that relate to procedure performance are collected and analyzed. For a manufacturing process these may include process parameters and test results on manufactured materials. For an analytical procedure they can include analytical results for controls, standards used during the analysis, and system suitability data. It’s important to note that the control samples are used to monitor the performance of the procedure and are not an indicator of the product performance or characteristics (FDA ISO 17025). For purposes of this appendix the term process will be used to refer to both a manufacturing process and an analytical procedure.

**Shewhart I-Chart**

To develop a control chart for individual observations, it is customary to set control limits at

$$\text{Process Mean} \pm 3 \times \text{Process Standard Deviation} \quad (32)$$

These limits are based on assuming the process data follow a normal probability distribution and that a range of 3 standard deviations about the mean contains roughly 99.7% of all the data. Given a sample of $Y_1, Y_2, \ldots, Y_n$ observations from a controlled process, the process mean (average) is estimated using the formula

$$\bar{Y} = \frac{1}{n} \sum_{i=1}^{n} Y_i \quad (33)$$

The standard deviation can be estimated in a couple of ways, but for an I-chart, best practice is to base the estimate on the moving range statistic (MR). This estimator considers the “short term” variability of the process and guards against limits that are too wide if an unexpected trend exists in the data. Specifically, the MR represents the average difference of successive observations and is defined as
\[ MR = \frac{1}{n-1} \sum_{i=2}^{n} |Y_i - Y_{i-1}| \]  
\[ (34) \]

and the estimator for the process standard deviation is

\[ \frac{MR}{d_2} \]  
\[ (35) \]

where \( d_2 \) is a constant that depends on the number of observations associated with the moving range calculation (\( m \)). In

\[ \text{equation (34)} \]  
\[ m = 2 \]  
\[ \text{since the range is based on adjacent observations. The value of } d_2 \text{ when } m = 2 \text{ is } 1.128. \]  

The upper control limit (UCL) and lower control limit (LCL) for the I-chart are then

\[ \begin{align*} 
\text{LCL} &= \bar{Y} - 3 \times \frac{MR}{d_2} \\
\text{UCL} &= \bar{Y} + 3 \times \frac{MR}{d_2} 
\end{align*} \]  
\[ (36) \]

To demonstrate, consider a sample of 20 observations with \( \bar{Y} = 31.2 \) and \( MR = 2.18 \). From \textit{equation (36)} the computed control limits are

\[ \begin{align*} 
\text{LCL} &= 31.2 - 3 \times \frac{2.18}{1.128} = 25.4 \\
\text{UCL} &= 31.2 + 3 \times \frac{2.18}{1.128} = 37.0 
\end{align*} \]  
\[ (37) \]

The associated I-chart is shown in 

\[ \text{Figure 1}. \]

\[ \text{Figure 1. I-chart for example data set.} \]

Detection of Out-Of-Control Results

After a control chart is constructed, out-of-control results are detected using either WECO or Nelson rules. The Nelson rules are provided in \textit{Table 10}. The relevance of these rules depends on the type of control chart. All eight rules can be applied to an I-chart, and selection of the particular rules depends on the desired sensitivity of the control process.

\textit{Table 10. Nelson rules for detection of out-of-control results}

<table>
<thead>
<tr>
<th>Rule</th>
<th>Description</th>
<th>Indication in an I-chart</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>One point exceeds either the LCL or UCL</td>
<td>One point is out of control</td>
</tr>
<tr>
<td>2</td>
<td>Nine points in a row on the same side of the center line</td>
<td>There is a mean shift in performance</td>
</tr>
<tr>
<td>3</td>
<td>Six points in a row steadily increasing or decreasing</td>
<td>A trend exists</td>
</tr>
<tr>
<td>4</td>
<td>14 points in a row alternating up and down</td>
<td>There is a negative correlation between neighboring points</td>
</tr>
<tr>
<td>5</td>
<td>Two out of three points on the same side of the mean and greater than two standard deviations away from the mean</td>
<td>A possible increase in assay variability</td>
</tr>
<tr>
<td>6</td>
<td>Four out of five points on the same side of the mean and greater than one standard deviation away from the mean</td>
<td>A possible increase in assay variability</td>
</tr>
<tr>
<td>7</td>
<td>15 points in a row within one standard deviation of the mean</td>
<td>A possible decrease in assay variability</td>
</tr>
<tr>
<td>8</td>
<td>Eight points in a row on both sides of the mean with none within one standard deviation of the mean</td>
<td>Non-random sample</td>
</tr>
</tbody>
</table>

Published on March 26, 2020
Figure 2 presents an I-chart for which a Rule 2 violation is observed because the last nine observations are all greater than the mean.

![I-chart](image)

**Figure 2.** Individual control chart with mean shift detected using Nelson rule 2.

ASTM E2587 (2016), Montgomery (2012), and Wheeler (2012) provide references for numerous control charts and example applications.

## APPENDIX 2: MODELS AND DATA CONSIDERATIONS

Statistical analysis involves models and assumptions associated with the reliability of fitting models to data. Models can be simple (e.g., a means model associated with a reportable value) or complicated (e.g., a nonlinear mixed effects model common in complex pharmaceutical settings). Assumptions monitored with residuals from the model fit include normality, constant variance, and independence. This appendix focuses on adequacy of models that are fit to analytical data, as well as data considerations such as significant digits, transformations, and outliers.

### Models

In statistics, a model represents a functional description of some property(s) of a population. The term population refers to the set of all possible values of an attribute. A model parameter, also referred to as a population parameter, is the true but unknown value of a property, which is typically the subject of the statistical inquiry.

A means model characterizes the center of a univariate population, and can be written as

\[
Y_i = \mu + E_i \tag{38}
\]

where \(Y_i\) is the \(i\)th observation in a sample of size \(n\) from the population, \(\mu\) is a model parameter representing the population mean, and \(E_i\) the error. This error represents the effect of all factors that explain why the measured value is not always equal to \(\mu\). Such factors typically include lot-to-lot variation in product or analytical method error. The means model is the basis of statistical inquiries related to a population mean, usually estimated by the sample mean

\[
\bar{Y} = \frac{1}{n} \sum_{i=1}^{n} Y_i \tag{39}
\]

with errors estimated by residuals \(R_i = Y_i - \bar{Y}\).

Another familiar model is the simple linear regression model. This model characterizes the linear trend in the population mean with some covariate \(X_i\) (e.g., time or dose), and can be written as

\[
Y_i = \alpha + \beta X_i + E_i \tag{40}
\]

where \((X_i, Y_i)\) is the \(i\)th observation in a sample of size \(n\) from the bivariate population, the parameters \(\alpha\) and \(\beta\) are the intercept and the slope, respectively, that defines the functional relationship and \(E_i\) the error. Note that \(\mu\) in model (38) has been replaced with \(\alpha + \beta X_i\) in model (40) to allow the mean to change as a function of \(X_i\). The parameters \(\alpha\) and \(\beta\) are estimated from sample data as was in model (38).

More complex models might be nonlinear, can include qualitative factors (e.g., analysts in a validation), or might include covariables which are random rather than fixed values (e.g., another measurement \(Z\) made together with \(X\)).

### Significant Digits

The number of digits used for calculations and the number of digits appearing in a reportable value should be considered separately. It’s important to record and carry more digits during calculation than will be reported. It is a good practice to perform
all statistical calculations with as many digits as practical. Rounding should be used only as a final step before reporting the result. Automation facilitates the acquisition of numerous digits, while databases should be designed to store data with enough digits in anticipation of further calculations from the data.

The number of digits reported can sensibly be based on the standard deviation of the reportable value. ASTM E29, USP General Notices, 7.20 Rounding Rules, and (23) provide guidance on rounding and determination of significant digits in a reported value.

**Transformation**

A transformation is a functional re-expression of a measurement in order to better represent a known scientific relationship or to satisfy the assumptions of a statistical model. Transformations can also be discovered empirically with a representative set of the data using residual plots. One particularly useful transformation with analytical data is the logarithmic (log) transformation described in the next section.

**LOG TRANSFORMATION**

Examples of transformations using scientific knowledge of the measurement system come from many biological systems. In particular, variation around the responses predicted by a means model is often proportional to the response. For these systems, it is useful to work with the log of the original response which will have nearly constant variance across the range of the response. The shape of the transformed distribution will also be more symmetric as shown in the lower panel of Figure 3. A log transformation can be conducted using any base including Napierian (base e), common (base 10), or base 2.

![Figure 3](image)

**Figure 3. A skewed log-normal distribution of potency (upper panel) and a symmetric normal distribution of log potency (lower panel).**

Another reason for using a log transformation is that it can change a nonlinear functional form in the original scale to something more easily modeled in the log scale. For example, a log transformation can be used to re-express a nonlinear first order kinetics model as a linear model.

Statistical measures associated with the center and the dispersion from a sample are described in Basic Statistical Principles and Uncertainty. These include the sample mean (Y) the sample standard deviation (S). These measures are meaningful when the data are approximately normally distributed and free of outliers. These measures may not be as meaningful when the normal distribution is not a good description of the data. To demonstrate, the top distribution in Figure 3 is skewed to the right. The greater values in the tail have the effect of pulling the mean to the right of where some would deem to be the “center” of the data. The lower distribution in Figure 3 shows the log-transformed responses of the top distribution. The top distribution is
called a log-normal distribution because the distribution of its log values is normal. Because of the symmetry of the normal curve, the sample mean and sample standard deviation are meaningful estimates of the center and dispersion of the transformed distribution.

The sample mean of log-transformed responses can be transformed back to the original scale. This back-transformation results in what is called the geometric mean (GM) on the original scale. More formally, let $Y_i$ represent a measured response on the original scale and $T_i$ the transformed value of $Y_i$. Then

$$T_i = \ln(Y_i)$$

$$\bar{T} = \frac{1}{n} \sum_{i=1}^{n} T_i$$

$$GM = \exp(\bar{T}) = \left( \prod_{i=1}^{n} \frac{1}{n} Y_i \right)^{1/n}$$  \hspace{1cm} (41)

The standard deviation of log-transformed responses ($S_T$) can likewise be back-transformed as $\exp(S_T)$. This term is referred to as the geometric standard deviation (GSD) by Kirkwood (1979). That is,

$$GSD = \exp(S_T)$$  \hspace{1cm} (42)

Because $S_T$ is non-negative, $GSD \geq 1$ and represents a fold-variation in the response scale. While a summary for arithmetically scaled responses can be written as $\pm S_T$, this might be summarized as GM $\times/\div$ GSD, or GM/GSD to GM $\times$ GSD for geometrically scaled responses. If for example GSD = 1.25 and GM = 1.0, a range might be summarized as $1.0/1.25 = 0.80$ to $1.0 \times 1.25 = 1.25$. It should be noted that this represents a 1-standard deviation range. A more appropriate range might be calculated in the log transformed scale (see below).

Kirkwood also defines the percent geometric coefficient of variation as

$$\%GCV = 100 \times (GSD - 1)\%$$  \hspace{1cm} (43)

An alternative measure of variability derived from the arithmetic moments of the log-normal distribution in the original scale is

$$\%CV = \sqrt{\exp(S_T^2) - 1} \times 100\%$$  \hspace{1cm} (44)

Numerically, $\%GCV$ and $\%CV$ of the log-normal distribution are close to each other when both are less than 20% (see Tan, 2005). Their use along with GSD should be clearly specified when reporting the measure of variability or intervals for log-normal data. Interpretation of these measures are described more fully in Biological Assay Validation (1033), Appendices, Appendix 1: Measures of Location and Spread for Log Normally Distributed Variables.

From equation (5) in Basic Statistical Principles and Uncertainty, a $100(1-\alpha)\%$ two-sided confidence interval on the mean in the log scale is

$$LB(T) = \bar{T} - t_{1-\alpha/2:n-1} \frac{S_{T}}{\sqrt{n}}$$

$$UB(T) = \bar{T} + t_{1-\alpha/2:n-1} \frac{S_{T}}{\sqrt{n}}$$  \hspace{1cm} (45)

where $n$ is the sample size and $t_{1-\alpha/2:n-1}$ is the $1 - \alpha/2$th quantile of the cumulative Student $t$ distribution having area $1 - \alpha/2$ to the left and $n - 1$ degrees of freedom.

The confidence interval on the geometric mean in the original scale is obtained from the bounds in equation (45) as

$$LB(Y) = \exp(LB(T))$$

$$UB(Y) = \exp(UB(T))$$  \hspace{1cm} (46)

Transformations other than logarithms may be considered for other types of data. For example, when working with proportions between 0 and 1 (or percentages between 0% and 100%), either the arcsine or logit transformation is useful. The arcsine transformation where $Y$ is represented as a proportion is

$$T = 2 \times \sin^{-1}(\sqrt{Y})$$  \hspace{1cm} (47)

and the logit transformation is

$$T = \ln\left(\frac{Y}{1-Y}\right)$$  \hspace{1cm} (48)
These transformations are particularly useful when a majority of the data are pushed against the upper bound of 1.0 or the lower bound of 0.0. Count data may be transformed using a square root or a log transformation of the count. Power transformations, the most common of which are Box-Cox transformations, are also useful re-expressions. These transformations are of the form

\[ T = \frac{Y^\lambda - 1}{\lambda}, \lambda \neq 0 \]

(49)

where \( \lambda \) is selected to best transform the data set to normality. Information on Box-Cox transformations is provided in Section 6.5.2 of the NIST/SEMATECH e-Handbook of Statistical Methods.

Regardless of the transformation, summary measures and intervals calculated in the transformed scale can be back-transformed to the original scale. In all cases the data should be examined to establish if the transformed measurements exhibit almost uniform variability and are approximately normally distributed.

**Assessing Model Adequacy**

All models involve assumptions about the processes that generate the data and the data itself. In addition to the assumed functional form, the distribution of the error terms, equations (38) and (40) is of primary importance. Typical assumptions are that the error terms are independent, normally distributed, and have constant variance across the range of responses. When these assumptions are reasonable, statistical models are usually readily interpretable and powerful (i.e., able to measure subtle effects with good precision and discrimination between groups). As attractive as any model might be, it is imperative to check for and address violations of the assumptions upon which these models rely. Assessing model adequacy is the process of verifying these assumptions.

There are both graphical and quantitative methods for assessing model adequacy. In many data analysis projects, there are multiple iterations of conversations between researchers and statisticians before selecting a final model. Topics to consider include appropriate transformations of the data, the treatment and design factors of interest, potential candidate models, and assessment of model fit.

Useful tools for assessing model fit include residual plots with both raw and studentized residuals, model-based outlier detection methods, and regression leverage and influence measures. Plots of residuals can be generated in several ways. The most common format is a plot of the residuals on the vertical axis, and the predicted response on the horizontal axis. When the observations on a residual plot increase or decrease in spread along the horizontal axis, this indicates violation of the assumption of constant variance. Any linear or non-linear trend in the residuals suggests the functional form of the model may not be correct, or that an important treatment factor is missing from the model. For example, a curved residual pattern may indicate the need for a quadratic term in the model. Additionally, residuals that fall outside the general cluster of points may be an indication of an outlier. As noted previously, some of these problems may be mitigated with an appropriate transformation.

Normality of the error terms is an especially important assumption if the model is used to predict future behavior. Graphical methods that can be used to monitor this assumption include dot plots, box and whisker plots, and normal probability plots (sometimes called quantile-quantile or QQ plots). These graphical tools are available in many common statistical software packages. Statistical tests of normality are described in Section 1.3.5 of the previously referenced NIST handbook and available in statistical software packages.

Lack of independence typically occurs when data are in some manner “batched” in groups. For example, measurements that are taken from the same plates on an assay are more similar than measurements recorded on other plates. This so-called intragroup correlation can be properly modeled by including a “batch” factor in the model to account for the correlation.

Care should be taken in the assessment of model assumptions. Statistical tests in particular are impacted by the size of the sample. For small samples such tests may be insensitive for detecting departures from the model assumptions. In contrast for large samples, they may detect an assumption violation even though visual assessment suggests the assumptions are reasonable. A combination of scientific understanding of the measurement process generating the data, graphical analyses and statistical tests can be used together to address model adequacy.

**Outliers**

Occasionally, observed analytical results are very different from expected analytical results. Aberrant observations are properly called outlying results. These outlying results should be documented, interpreted, and managed. Such results may be accurate measurements of the property being measured but are very different from what is expected. Alternatively, due to an error in the analytical system, the results may not be typical, even though the property being measured is typical. A first defense against obtaining an outlying analytical result is application of an appropriate set of system suitability and control rules (see Appendix 1: Control Charts).

When an outlying result is obtained, systematic laboratory and process investigations are conducted to determine if an assignable cause can be established to explain the result. Factors to be considered when investigating an outlying result include human error, instrumentation error, calculation error, and product or component deficiency. A thorough investigation should consider the precision and accuracy of the procedure, the USP or in-house Reference Standard and controls, process and analytical trends, and the specification limits. If an assignable cause due to the analytical procedure can be identified, then retesting may be performed on the same sample, if appropriate, or on a new sample. Based on the documented investigation, data may be invalidated and eliminated from subsequent calculations.
“Outlier labeling” is informal recognition of outlying results that should be further investigated with more formal methods. Outlier labeling is most often performed visually with graphical techniques such as residual plots, standardized residual plots, or box and whisker plots. “Outlier identification” is the use of statistical significance tests to confirm that the values are inconsistent with the known or assumed data distribution. The selection of the correct outlier identification technique often depends on the initial recognition of the number and location of the values.

A simple example is presented to demonstrate this process. An analytical procedure requires measurements from three vials of liquid drug product which are used to provide a reportable concentration value (mg/ml) for the lot from which the vials were selected. When measuring the third vial, the analyst noted a slight deviation in the sample preparation which was not discussed in the protocol. The three measurements are reported in Table 11. Vial 3 is the vial in question.

Table 11. Concentrations for three vials of drug product

<table>
<thead>
<tr>
<th>Vial</th>
<th>Concentration (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>49.9</td>
</tr>
<tr>
<td>2</td>
<td>49.8</td>
</tr>
<tr>
<td>3</td>
<td>51.8</td>
</tr>
</tbody>
</table>

The residual plot for the mean model described in equation (38) is shown in Figure 4. Here the residual is the measured value minus the sample mean of the three vials (50.5 mg/ml).

The residual for vial 3 visually resides far from the other two values and is accordingly labeled as an outlier.

One statistical test that can be used to determine if vial 3 can be identified as an outlier is due to Dixon (1950, 1951). This test is based on a ratio of differences between the observations. For this particular application where interest is in determining if the maximum value is an outlier, a single test statistic is computed and compared to a critical value based on a normal probability distribution. The minimum value in the data set is 49.8 mg/ml, the middle value is 49.9 mg/ml, and the maximum value is 51.8 mg/ml. The test statistic is defined as

\[
\frac{\text{Maximum} - \text{Middle}}{\text{Maximum} - \text{Minimum}} = \frac{51.8 - 49.9}{51.8 - 49.8} = 0.95
\]

The calculated value in equation (50) is then compared to a table of values based on the distribution of order statistics for a normal probability distribution. The critical value that must be exceeded to be identified as an outlier with three values using a type 1 error rate of 0.05 and assuming a normal distribution is 0.941. Since the computed value of 0.95 exceeds 0.941, the measurement of vial 3 is identified as an outlier. Actions to be taken will depend on results of further investigations.

As noted, this particular version of the Dixon test requires an assumption of normality which cannot be verified with such a small sample. Rather, one would need to rely on previous measurements made with the procedure on previous process lots to support this argument. In general, the critical value as well as the ratio that one constructs for the Dixon test depends on the number of measurements in the data set and the type 1 error rate. A complete set of critical values for sample sizes less than 30 are available in Böhrer (2008).

As noted previously, the process of identifying a statistical outlier generally requires scientific support for an assignable cause. For the applications performed in an analytical lab, candidate outlier tests are typically univariate. Two questions to consider when selecting a method are

1. Can the distribution be assumed to be normal, or should a test be applied that does not require this particular distributional form?
2. Do we suspect more than one outlier, and which observation(s) have been labeled?

With regard to question 1, outlier tests can be categorized as either parametric (model-based) or non-parametric. The parametric structure selected by such methods is typically the normal distribution. Question 2 considers whether there is one
APPENDIX 3: EQUIVALENCE AND NONINFERIORITY TESTING

General Notices describes the need to produce comparable results to the compendial method. Several options were identified to address this as noted in Hauck et. al. (2009). Among these was performance equivalence. Performance equivalence is used to establish the equivalence of the two procedure means, and noninferiority of the new procedure variability to that of the old procedure, as the basis for demonstrating comparability between two procedures.

The article goes on to describe an approach for demonstrating comparability using statistical hypothesis testing. This appendix describes the general principles of statistical hypothesis testing, as applied to equivalence testing of procedure means and noninferiority testing of procedure variabilities.

In classical statistical hypothesis testing, there are two hypotheses, the null and the alternative. For example, when comparing a new and an old procedure, the null may be that two means are equal and the alternative that they differ. This may be expressed as

\[ H_0: \mu_N = \mu_O \]
\[ H_a: \mu_N \neq \mu_O \] (51)

or equivalently

\[ H_0^ ': \mu_N = \mu_O = 0 \]
\[ H_a^ ': \mu_N - \mu_O \neq 0 \] (52)

where \( \mu_N \) and \( \mu_O \) are the means for the new and old procedures, respectively.

With this classical approach, one rejects the null hypothesis in favor of the alternative if the evidence is sufficient against the null. In such a case we accept the alternative hypothesis that the means are different. Because of this interpretation, this is sometimes called a difference test.

A common misinterpretation is to conclude that failure to reject the null hypothesis in a difference test is evidence that the null is true (i.e., the means are equal). Actually, failure to reject the null just means the evidence against the null was not sufficient to claim the means are different. This might occur if the variability is large, or the number of determinations too small to detect a difference in the means.

Thus, when one seeks to demonstrate equivalence of procedure means, it is necessary to place the claim of equivalence in the alternative hypothesis. A statistical test for an alternative hypothesis of equivalence is referred to as an equivalence test. It is important to understand that “equivalence” does not mean “equality.” Equivalence should be understood as “sufficiently similar” for the use of the new procedure. The definition of “sufficiently similar” is something to be decided a priori based on scientific considerations, and becomes the basis of the alternative hypothesis. Chatfield and Borman (2009) offer some helpful suggestions for this process.

As a specific example, suppose it is decided a priori that to be considered equivalent, the means of two procedures can differ by no more than some positive value, \( d \). This value is commonly called the equivalence margin. The hypotheses for the equivalence test are then

\[ H_0^ e: |\mu_N - \mu_O| \geq d \]
\[ H_a^ e: |\mu_N - \mu_O| < d \] (53)

Note the alternative hypothesis is actually two individual one-sided hypotheses:

1. \( H_{a1}^ e: \mu_N - \mu_O < d \), and
2. \( H_{a2}^ e: \mu_N - \mu_O > -d \).

For this reason, this testing procedure is referred to as two one-sided tests (TOST). As one-sided tests, each can be addressed with a type I error rate of \( \alpha \) (typically, but not necessarily, 0.05). The TOST is often conducted by rejecting the null hypothesis in favor of the alternative hypothesis if the 100\( (1 - 2\alpha) \% \) two-sided confidence interval (typically, but not necessarily 90\% ) is entirely contained in the range \((-d, +d)\). When the null is rejected, we conclude that the two procedures are equivalent in their means.

Performance equivalence is not restricted to demonstrating equivalence of procedure means. A laboratory might want a new procedure to have equivalent or better variability as the old procedure. This requires a one-sided test because if the new procedure were to have a lesser variability, this would clearly be acceptable. What one needs to ensure is that the new procedure does not result in an important increase in variability. Thus, variability comparisons are conducted as one-sided noninferiority tests.

Similar to an equivalence test for means, a noninferiority test for variabilities places the desired relationship between procedure variabilities in the alternative hypothesis. Due to the statistical properties of standard deviations, an appropriate parameter for comparison is the ratio, \( \sigma_N/\sigma_O \), where \( \sigma_N \) and \( \sigma_O \) represent the standard deviations of the new and the old procedures, respectively.
Suppose it is determined a priori that for the procedure to be fit for use, the standard deviation of the new procedure can exceed that of the old procedure by no more than a factor $k \geq 1$. The factor $k$ is called the noninferiority margin. The hypotheses associated with the noninferiority test are

\[ H_0: \frac{\sigma_N}{\sigma_0} \geq k \]
\[ H_0: \frac{\sigma_N}{\sigma_0} < k \]

Unlike the equivalence test of means, the noninferiority hypothesis is a single hypothesis which can be addressed with a level $\alpha$ (typically, but not necessarily, 0.05). In order to perform the test, the null hypothesis is rejected in favor of the alternative hypothesis if the $100(1 - \alpha)$% upper one-sided confidence bound on $\sigma_N/\sigma_0$ is less than $k$. When the null hypothesis is rejected, it is concluded that the variability of the new procedure is noninferior to that of the old procedure.

Hauck, et al. offers other options to address the standard of “equivalent or better”:

1. minimum performance requirements for acceptable procedures,
2. results equivalence, and
3. decision equivalence.

The option of minimum performance requirements has evolved into the concept of the analytical target profile (ATP) which has been introduced in Pharmacopeial Forum (Barnett et al. 2016). Results equivalence is addressed using the intra-class correlation coefficient or the concordance correlation coefficient. A tolerance interval approach using total variability is likewise used to address results. Decision equivalence relates to dichotomous outcomes such as pass/fail, and can be addressed through the kappa coefficient or receiver operating characteristic curves. Using these options (as with performance equivalence), care must be taken to properly formulate the statistical hypotheses and to address the comparison through meaningful acceptance criteria.

While this appendix has highlighted approaches for establishing procedure comparability, these apply to other scenarios involving comparisons of two groups; e.g., procedure transfer or standard qualification. Placement of the claim one desires to support into the alternative hypothesis results in an appropriate statistical conclusion.

Although the benefits of equivalence testing are apparent, in some situations one may not be able to collect a sufficient sample size to provide the necessary power to establish equivalence. In such a situation, use of the difference test may be the only option. However, one is reminded that failure to reject the null hypothesis of equality is not evidence that the procedure means are equal. A confidence interval should nonetheless be reported to communicate the difference of means between the two procedures.

**APPENDIX 4: THE PRINCIPLE OF UNCERTAINTY**

While this chapter has concentrated on statistical studies which are performed using measurement data, the principles and practices are identical to those in the field of metrology. These are unified by a common understanding of the concept of uncertainty. This appendix introduces concepts related to the metrological principle of measurement uncertainty and unify these with the practices described for the scientific method.

The understanding of study uncertainty is not new to the pharmaceutical industry and has been employed more broadly throughout industries that make decisions from studies using measurements. The study of measurement uncertainty falls formally into the field of metrology. A measurement process like a study is designed to reduce uncertainty in order to make a more informed decision. No measurement or study result can provide exact knowledge. Proper interpretation and treatment of analytical data requires an understanding of the inherent sources of uncertainty in measurement outcomes and their impact on the information they provide. Recognition of the principles of uncertainty facilitates this understanding, as described by the Joint Committee for Guides in Metrology in the Guide to the Expression of Uncertainty in Measurement (GUM).

Results from all studies, including quality control testing are uncertain. Uncertainty arises from sources of variability inherent in the measurement process, as well as from statistical sampling and study factors. The principles from the field of metrology are consistent with the statistical principles described in this chapter and provide further insight into the quantification of uncertainty from studies supported by measurements.

At the core of these principles is an understanding of risk. More specifically, this understanding considers the risks of making incorrect decisions based on studies utilizing measurements. The consequences of these risks can be minor or significant, and thus should be factored into considerations related to the design of a measurement system, the design of studies using the measurement system, and the interpretation of study results. The concepts of Target Measurement Uncertainty (TMU) and the study objective can be unified as a basis for managing the risks associated with making decisions from studies. In fact, TMU is a special case of a study hypothesis which drives the design of all studies using analytical measurements.

To increase knowledge, two of the fundamental forces of metrological and statistical thinking are the desire to minimize the uncertainty in the measured value (an indication of the quantity being measured) and to ensure all sources of uncertainty have been evaluated and mitigated. In metrology the quantity intended to be measured is termed the measurand. This is called a population parameter in the broader sense of a study. Measurement or parameter uncertainty quantifies one’s doubt about the true value that remains after making a measurement or estimating a parameter.

While the metrological concept of measurement uncertainty applies exclusively to a reportable value, this can be aligned with the concept of study uncertainty by viewing the quality control process as a study of a commercial lot. Employing the steps of the scientific method, the study of the commercial lot has an objective which can be formulated as a hypothesis test.
where \( \mu \) is the commercial lot mean and LSL and USL are the lower and upper specification limits respectively. The study can be designed using blocking and replication to satisfy the TMU, which should be such as to minimize the risks associated with the object of the testing (i.e., to support the alternative hypothesis, \( H_a \)). As part of study conduct, sampling and randomization can be utilized to mitigate the risks due to the introduction of bias. Finally, and perhaps most importantly, the data should be analyzed and reported with acknowledgement of the uncertainty in the reportable value.

**Metrological Principles Specific to Measurement Uncertainty**

The reliability of study results are only as good as the fitness for use of the measurement process used to generate data for the study. The metrological concept of measurement uncertainty helps to ensure fitness for use. This and other principles are worth noting as a fundamental way to view a measurement process.

Figure 5 represents several potential sources of random variation in a measurement process, which result in the combined standard uncertainty (the estimated standard deviation of the measurement). An example of inherent random variation is when the same chromatogram is given to several different analysts for peak integration. Slightly different values will be obtained which might also be affected by a laboratory’s choice of software. In addition, the definition of the measurand can never be complete. This is known as definitional uncertainty or uncertainty of knowledge. Ideally the measurand is defined sufficiently so that the definitional uncertainty is relatively small when compared to the combined standard uncertainty. An example of lack of knowledge is when a component of the measurement process has associated uncertainty. For example, one might purchase a pH standard solution that is certified as \( pH = 7.00 \pm 0.02 \) where the 0.02 is the expanded uncertainty in the assigned value of the standard solution. Expanded uncertainty is a measure of uncertainty that defines an interval about the measurement result \( y \) within which the value of the measurand \( Y \) can be confidently asserted to lie.

GUM notes the evaluation of measurement uncertainty is neither a routine task nor a purely mathematical one. Judgment is essential in choosing which uncertainty components (i.e., potential causes of measurement uncertainty) to consider in procedure development, qualification, and measurement uncertainty evaluation. For example, when preparing a 1 mg/L solution, the process by which it is prepared can influence the final concentration. The analyst knows it would not be wise to weigh 1 mg of the substance followed by dilution to 1 L. Instead, recognizing the limits of typical analytical balances, a more precise result would be achieved by weighing 100 mg of substance and then serially diluting to the required concentration. Measurement uncertainty arises from many sources including differences in instruments, mathematical algorithms, and analysts. A tutorial sampling of typical uncertainty components is provided in Table 12.

| Variability due to analytical procedure design | Effect of the sample amount or volume |
| Variability due to measurement process | Carry-over effects in the auto sampler |
| Variability due to analysts | Effect of the sample storage conditions |
| Variability due to algorithms | Failure to recognize ruggedness factors |
| Variability due to sample | Effect of the manual peak integration |

Table 12. A tutorial list of uncertainty components in analytical laboratory practice
A detailed discussion of measurement uncertainty in the pharmaceutical industry that expands upon the metrological principles introduced here and provides detailed definitions is provided in Weitzel et al. (2018). In addition, a worked example for a drug substance is provided in Weitzel et al. (2017).

**APPENDIX 5: BAYESIAN INFERENCE**

When describing statistical intervals in Section 4, Basic Statistical Principle and Uncertainty, it was noted that one can utilize a Bayesian approach to derive an interval which contains, with probability $100 \times (1 - \alpha)\%$ the true value of the population mean. This is important because it returns a statement that the laboratory frequently wishes to make. This section will describe Bayesian inference and contrast it with frequentist inference which is more commonly understood throughout the pharmaceutical industry. Frequentist theory bases inferences on probability statements about statistics, while Bayesian inference is based on probability statements about population parameters. Population parameters are the unknowns that appear in statistical models (e.g., means, variances, difference of means) and statistics are summary measures or estimates based on data (e.g., parameter estimates). Frequentist inference regards parameter values as fixed and unknowable whereas Bayesian inference models their uncertainty using probability distributions. For instance the statement “there is a 95% probability that the difference in population means is between −0.1 to 0.1” is meaningless from a frequentist viewpoint, but reasonable from a Bayesian perspective. The Bayesian formulation offers a way for scientists needing to make risk based decisions. Bayesian inference can also incorporate prior information about statistical parameters together with the sample data to update what is known about a parameter. The ability to incorporate justified prior information potentially leads to better decisions when a study size is small, or when a factor is not adequately represented in the study design.

The purpose of this appendix is to provide a basic introduction to Bayesian inference applied to statistical studies and to analytical measurements. Gelman et al. (2013) provides a source for more information.

**Parameter Uncertainty versus Sampling Variability**

Parameters are unknown hypothetical or population quantities, such as the mean or standard deviation of a population, or the difference in means between procedures. While unknown a parameter can be estimated. The estimation of a parameter and the inherent uncertainty of that estimation is the basis of Bayesian thinking.

Statistics are observed quantities or summaries of observed quantities in a sample taken from a population or process of interest. Examples of statistics include an analytical result (a measurement), a sample mean, a sample standard deviation, a difference in observed means between procedures, or their estimated confidence bounds. On repeated sampling of the population, the observed values of statistics will differ because of sampling variability.

Frequentist statistical methodology considers parameters to be fixed values that do not change. It employs probability theory to model the sampling variability of statistics randomly obtained from the population. These sampling distributions are then used to make inferences about the fixed value of the parameter. A common frequentist methodology is the calculation of a confidence interval. The process of computing a 95% confidence interval ensures that the realized interval will contain (or cover) the unknown parameter 95% of the time on repeated use.

The 95% refers to the reliability of the methodology (i.e., its coverage), and not the probability that the parameter falls within the interval.

For example, suppose a computed 95% confidence interval on a mean is from 980 to 990 mg/g. It is not correct to state there is a 95% probability that the population mean is between 980 and 990 mg/g. To associate a probability with a fixed interval such as 980 to 990 mg/g, one must assume uncertainty is associated with the underlying parameter (i.e., it is not a fixed quantity). Rather, the 95% description of the confidence interval means that the interval will correctly contain the true parameter value in 95% of repeated sampling applications from the population. The 95% refers to the success rate of the sampling process and not the parameter (which is assumed fixed).

Bayesian statistical methodology considers a parameter value to be uncertain (not fixed), and models its likely levels using a probability distribution. It extends frequentist statistical methodology, using probability theory to model both the sampling variability of statistics and the decision maker’s uncertainty associated with parameters. Bayesian models are sometimes called “complete” probability models because they quantify the uncertainty associated with the parameters of interest, given the assumed sampling variability of the observed statistics, any relevant prior information, and the observed data. For instance, it is correct to say that a given Bayesian 95% credible interval (Bayesian analogue of the confidence interval) contains the value of a specific parameter of interest with 95% probability (conditional on the observed data and other modeling assumptions).

The same principles apply to the Bayesian analogues of frequentist tolerance and prediction intervals. Unlike frequentist interval methodology in which the probability level must be fixed in advance (e.g., 95%) and the resulting interval is random, Bayesian methodology offers the opportunity to fix the interval in advance, and estimate the probability that the parameter value lies within that interval. Such an application is extremely useful for determining the probability that an analytical procedure will provide a signal outside a given range.

**Prior and Posterior Distributions**

Both frequentist and Bayesian methodologies express models using probability distributions. Both use the same model for sampling variability known as the likelihood. The particular likelihood model choice is based on prior knowledge concerning statistical variability.

Bayesian inference also requires a probability model for parameter uncertainty, prior to observing the data, called the prior distribution. As with the likelihood, the prior distribution is a choice based on prior data, reliable knowledge, or common sense (e.g., the values of many parameters, such as a standard deviation, must be positive). Bayesian methodology requires care to
assure that the chosen prior distributions are scientifically justified and do not unduly influence the inference. Use of appropriately justified knowledge of a prior distribution can potentially reduce sample size requirements for decision making. However, when there is little available theory, historical data, or expert knowledge available, prior distributions can be constructed that give minimal preference to any particular parameter value, and thus have minimal impact on the inference. Such prior distributions are often referred to as “non-informative”. When non-informative prior distributions are employed, inferences typically agree with the frequentist counterparts since both are solely dependent on the likelihood.

Bayesian methodology combines likelihood and prior distributional models with observed data to produce an updated distributional model for parameter uncertainty called the posterior distribution. The posterior distribution provides the probability that the population parameter value lies within any interval of interest. Such intervals are called credible intervals. When certain classes of non-informative prior distributions (e.g., a Jeffrey prior used with a normal likelihood) are employed, a Bayesian credible interval can be calculated from the posterior distribution, and may sometimes be numerically equal to the corresponding traditional confidence interval. However, as previously noted, the interpretations of these intervals are different. The probability associated with the credible interval quantifies uncertainty in an estimated parameter value conditional on observed data, while the probability associated with the confidence interval quantifies the probability of coverage of the estimated parameter on repeated estimation over many data sets.

From the Bayesian perspective, all knowledge about the parameter of interest is based on the posterior distribution. The posterior distribution from a previous study can inform the prior distribution for a subsequent study. Updating the prior distribution in this manner as new data become available, provides a paradigm for knowledge building, and thus a statistical basis for applying prior knowledge during pharmaceutical development (see ICH Q8(R2), Pharmaceutical Development).

The posterior distribution of parameters may also be re-combined with the likelihood to obtain a posterior predictive distribution of future observed data or statistics. As with the posterior distribution, the Bayesian perspective bases all knowledge about future values on this posterior predictive distribution, which can be used to construct Bayesian analogues of frequentist tolerance and prediction intervals. Unlike the frequentist analogues, the Bayesian intervals do not require a pre-specified fixed probability level. A posterior predictive distribution can be used, for example, in estimating the probability of occurrence of future out-of-specification results.

An Illustrative Example

Consider an analytical procedure for strength of drug product. The output of the procedure is a reportable value (mg/g) that estimates the mean strength, \( \mu \), for the tested lot of drug product. For the lot to be considered safe and effective, \( \mu \) must be between 980 and 1020 mg/g. The observed reportable result, \( Y \), is 1010 mg/g.

A typical rule used for disposition is to release the lot if \( 980 \leq Y \leq 1020 \). However, this rule is based on an observed reportable result that includes measurement error from the analytical procedure. What we really want to know is whether the population parameter value lies within any interval of interest. Such intervals are called credible intervals. When certain classes of non-informative prior distributions (e.g., a Jeffrey prior used with a normal likelihood) are employed, a Bayesian credible interval can be calculated from the posterior distribution, and may sometimes be numerically equal to the corresponding traditional confidence interval. However, as previously noted, the interpretations of these intervals are different. The probability associated with the credible interval quantifies uncertainty in an estimated parameter value conditional on observed data, while the probability associated with the confidence interval quantifies the probability of coverage of the estimated parameter on repeated estimation over many data sets.

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An Illustrative Example

Consider an analytical procedure for strength of drug product. The output of the procedure is a reportable value (mg/g) that estimates the mean strength, \( \mu \), for the tested lot of drug product. For the lot to be considered safe and effective, \( \mu \) must be between 980 and 1020 mg/g. The observed reportable result, \( Y \), is 1010 mg/g.

A typical rule used for disposition is to release the lot if \( 980 \leq Y \leq 1020 \). However, this rule is based on an observed reportable result that includes measurement error from the analytical procedure. What we really want to know is whether the population parameter value lies within any interval of interest. Such intervals are called credible intervals. When certain classes of non-informative prior distributions (e.g., a Jeffrey prior used with a normal likelihood) are employed, a Bayesian credible interval can be calculated from the posterior distribution, and may sometimes be numerically equal to the corresponding traditional confidence interval. However, as previously noted, the interpretations of these intervals are different. The probability associated with the credible interval quantifies uncertainty in an estimated parameter value conditional on observed data, while the probability associated with the confidence interval quantifies the probability of coverage of the estimated parameter on repeated estimation over many data sets.

From the Bayesian perspective, all knowledge about the parameter of interest is based on the posterior distribution. The posterior distribution from a previous study can inform the prior distribution for a subsequent study. Updating the prior distribution in this manner as new data become available, provides a paradigm for knowledge building, and thus a statistical basis for applying prior knowledge during pharmaceutical development (see ICH Q8(R2), Pharmaceutical Development).

The posterior distribution of parameters may also be re-combined with the likelihood to obtain a posterior predictive distribution of future observed data or statistics. As with the posterior distribution, the Bayesian perspective bases all knowledge about future values on this posterior predictive distribution, which can be used to construct Bayesian analogues of frequentist tolerance and prediction intervals. Unlike the frequentist analogues, the Bayesian intervals do not require a pre-specified fixed probability level. A posterior predictive distribution can be used, for example, in estimating the probability of occurrence of future out-of-specification results.
A Comparison of Frequentist and Bayesian Methods

Both frequentist and Bayesian approaches to inference are useful. Frequentist approaches are widely available, straightforward, and offer the reliability of known coverage probability. Bayesian approaches can be used to quantify the uncertainty in parameters of interest which can support quantitative risk based decision making. While often more technically challenging to apply, Bayesian MCMC methodology can often be applied to problems that are intractable by frequentist approaches. When informative prior distributions can be justified, Bayesian methods may require smaller samples sizes for decision making than frequentist statistical methods. Table 13 provides a comparison of some characteristics from both frequentist and Bayesian perspectives.

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<tr>
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<tr>
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APPENDIX 6: REFERENCES

BOVINE SERUM

INTRODUCTION

Bovine serum is the liquid fraction of clotted blood, obtained from an ox (Bos taurus, among others), that has been depleted of cells, fibrin, and clotting factors. Since the advent of modern cell culture, manufacturers of biological products have used bovine serum extensively as a cell culture growth supplement. Its rich nutritional composition of proteins, growth factors, hormones, amino acids, vitamins, sugars, lipids, trace elements, and other components supports a broad range of cell culture applications in research and commercial manufacture of vaccines, natural source and recombinant biologics (hereafter biologics), engineered tissues, and other emerging cell-based therapeutic products intended for human or veterinary use. The predominant type of serum used in research applications is Fetal Bovine Serum (FBS). Calf serum (from newborn and older animals) is used much less frequently, but because of its lower cost it may be widely used in commercial manufacturing.

As is the case with other animal-derived products, bovine serum carries a potential risk of introducing extraneous agents into cell culture. Serum manufacturers and regulators must adopt rigorous sourcing and testing procedures and strict processing and production guidelines to ensure the quality of bovine serum.

The objective of increasing the quality and safety of biologics produced with bovine serum, coupled with attempts to mitigate regulatory burden, have caused developers to investigate alternatives to serum supplementation, resulting in application-specific serum-free medium formulations. Although it is recognized that bovine serum should be avoided when there is an option to use serum-free medium, there are cases where this is technically impossible or impractical.

This chapter describes issues related to sourcing, production, and characterization of bovine serum to ensure its safe use. A list of relevant regulatory and guidance documents is presented in Appendix 1. Serum manufacturers and serum end users (manufacturers of biological products) should consider and apply as needed the controls and procedures outlined in this chapter to ensure the safe use of bovine serum components in research and pharmaceutical manufacturing.
Types of Bovine Serum

- FBS is obtained from the fetuses of healthy, prepartum bovine dams that had been deemed fit for human consumption through ante- and postmortem inspection by licensed veterinarians. It is collected in government-inspected and -registered slaughterhouses.
- Newborn calf serum (also known as newborn bovine serum) is obtained in government-inspected and -registered slaughterhouses from animals aged less than 20 days.
- Calf serum is obtained in government-inspected and -registered slaughterhouses from animals aged between 20 days and 12 months.
- Donor bovine serum (also known as donor calf serum) is obtained by the repeated bleeding of donor animals from controlled government-inspected and -registered donor herds. The animals are 12–36 months old.
- Adult bovine serum is obtained in government inspected and -registered slaughterhouses from cattle older than 12 months that are declared fit for human consumption.

BOVINE SERUM: HISTORY AND TYPES OF USE

History of Bovine Serum Use

Animal serum and other complex biological materials have been employed in the cultivation of mammalian cells for approximately 100 years. Several factors led to the wide adoption of bovine serum as a standard tissue culture supplement. In comparison to serum from other animal species (horse, goat), bovine serum is easily sourced, and thereby more affordable. Many investigators choose to use fetal serum in their experimental systems because of concerns associated with antibodies present in newborn and adult serum that could cross-react with cells in culture and cause cell lysis through complement-mediated pathways. To eliminate that concern, heat was introduced to inactivate complement that was potentially present in the serum. Studies of FBS undertaken in the 1950s on the cultivation of low-density human cells to elucidate mechanisms of cell growth found that (1) the albumin component may serve as a carrier of essential small molecules; (2) fetuin, a glycoprotein present at high levels in the alpha globulin fraction, facilitates cell attachment and stretching; and (3) fetuin markedly inhibits trypsin, and this antiproteolytic activity may play a role in the ability of fetuin to stimulate cell growth.

In the 1960s and 1970s, serum supplementation of tissue culture media became the norm, thus facilitating biomedical research as well as the first large-scale vaccine manufacturing processes. Serum supplementation reduced the requirement for optimizing medium formulations for different cell types. FBS was shown to provide a variety of polypeptide growth factors. Albumin promoted cell growth presumably because of its abilities to function as a carrier protein for small molecules or lipids, to bind metal ions, to serve as a pH buffer, and to protect cells against shear. Similar functions were found for other serum components such as transferrin, hormones, and other serum-derived attachment factors such as fibronectin, vitronectin, and laminin.

Uses of Bovine Serum

Serum is a complex mixture of macromolecules that is required for cell growth and virus production, and its use as a raw material presents a number of challenges. These include its batch-to-batch composition and the risk of contamination by adventitious agents. The development of serum-free media has replaced serum in some new biotechnology manufacturing applications, but many cell lines used in manufacturing have not been adapted to these serum-free media. Regulatory constraints and scientific challenges generally make it impractical to alter existing manufacturing processes in which serum is used as a raw material.

FBS sometimes is required in cell and tissue bioprocessing, which often involves the cultivation of cells from tissue explants and biopsies. Some bioprocesses may also require the maintenance of specific cellular characteristics during cultivation. FBS often appears to facilitate such procedures and may affect the biological behavior of fastidious cell types. FBS has been shown to affect the transcription of developmentally important genes, apoptosis, and apoptosis-related gene expression, and to provide neuroprotective and antioxidative factors, all of which may be beneficial to the survival and development of cells in culture. Therefore, FBS will continue to play an important role as a cell culture supplement for production of cell- and tissue-based therapies.

In most viral vaccine manufacturing processes the media used for cell culture expansion and virus infection/production are supplemented with different types of serum at different concentrations. In these processes, bovine serum helps generate a mass of cells in an optimal physiological state for efficient viral replication.

BOVINE SERUM HARVESTING AND PRODUCTION

Blood Collection

For all types of bovine sera, blood should be collected in government-inspected and -registered premises (slaughterhouses, abattoirs, and donor farms). Blood should be collected by trained operators following the written procedures approved by the serum manufacturer and using either single-use disposable collection devices or reusable collection equipment for which cleaning procedures have been validated.
DONOR BOVINE SERUM

For each lot of serum from donor animals, serum manufacturers should ensure traceability to the donor herd of origin via production records and animal health and origin certificates. Donor animals are subjected to regular veterinary inspections and are bled multiple times following established procedures. Animals introduced into the herd should be traceable by source, breeding, and rearing history. Collectors should introduce new animals into the herd following specified and approved procedures that include prepurchase animal inspection and testing, proper transportation, a quarantine period, veterinary examination and testing during the quarantine period, and animal release criteria from quarantine to serum production. The collectors should not vaccinate donor animals for bovine viral diarrhea (BVD). Collectors should test animals for any agent and antibody from which the herd is claimed to be free.

NEWBORN CALF SERUM, CALF SERUM, AND ADULT BOVINE SERUM

Certificates of animal health and origin and/or serum production records should ensure that serum manufacturers can trace bovine serum derived from slaughtered animals back to the abattoir. Serum manufacturers should require abattoirs to maintain documentation of the origin of animals for slaughter. Blood should be collected from animals that have been slaughtered, for human consumption, in abattoirs inspected by the competent authority of the country of origin. Inspectors should routinely inspect animals both antemortem and postmortem to check for the clinical appearance of infections and parasitic diseases and other animal health-related problems or conditions. The animals must be free of clinical evidence of infectious diseases at the time of slaughter. Blood collection procedures must be in place to prevent cross-contamination with other tissues and body fluids and the surrounding environment. The standard procedure of slaughter consists of an approved method of animal stunning followed by exsanguination.

FETAL BOVINE SERUM

FBS product specifications and test procedures are presented in the proposed general chapter Fetal Bovine Serum—Quality Attributes and Functionality Tests (90). Serum manufacturers should collect fetal bovine blood from bovine fetuses whose dams have been slaughtered. The dams must have been deemed fit for human consumption and must have been slaughtered in abattoirs that were inspected by the competent authority of the country of origin. Inspectors should examine all animals both antemortem and postmortem to check for the clinical appearance of infections and parasitic diseases and other animal health-related problems or conditions. The animals must be free of clinical evidence of infectious diseases at the time of slaughter. The uterus is removed and transported to a dedicated space for fetal bovine blood harvest, where blood collection personnel evaluate the fetus for signs of fetal death, including bloating, skin discoloration, odor, deformation, and hair sloughing. Collectors also should check the amniotic fluid for color, quantity, and clarity. Serum manufacturers should collect blood from acceptable fetuses by cardiac puncture into a closed collection system under conditions designed to minimize microbial contamination. Manufacturers should have in place procedures that will prevent cross-contamination with other fetal tissues and bodily fluids and the surrounding environment.

Serum Harvesting and Processing

Trained personnel should perform serum separation (harvesting) and further processing activities following written and approved procedures. Serum is first separated and pooled, followed by filtration and filling into clean and disinfected containers. If the serum is subjected to one or more virus inactivation treatments in the production process, serum manufacturers should validate the virus inactivation processes against a range of relevant viruses. It is recommended that bovine viral diarrhea virus (BVDV) be included in any virus validation study because it is ubiquitous.

SERUM SEPARATION AND HARVESTING

Bovine blood should be processed and serum separated (harvested) in such a way as to minimize bacterial and mycoplasmal contamination, which in turn minimizes endotoxin levels in serum product. Gentle, quick blood processing helps to minimize hemolysis, further enhancing the quality of the serum product. After collection, blood is first allowed to clot for a specified period of time and under controlled conditions, then centrifuged in a refrigerated centrifuge. Serum is then removed from the clot, typically by centrifugation; pooled and mixed in a pooling vessel; transferred to labeled containers; and frozen, unless it is filter-sterilized immediately. Serum manufacturers should describe each process step and carry out serum processing activities, including sample collection and in-process quality control testing, following the manufacturer’s approved procedures.

POOLING BEFORE FILTRATION

Because limited amounts of blood can be collected from individual animals, serum manufacturers pool the raw serum from many animals in order to create commercial-sized lots. Serum is pooled, after raw serum thawing and before filtration, in a pooling vessel and mixed at a controlled mixing rate and temperature. Pools or lots of donor bovine serum may consist of many separate collections from individual members of the herd. Lots of FBS may consist of pooled serum from thousands of animals. Serum manufacturers should describe each prefiltration pooling process step and should carry out serum thawing, prefiltration pooling, and mixing activities following the manufacturer’s approved procedures.

FILTRATION

Pooled serum is mixed and aseptically passed through filters of pore size 0.2 µm or smaller, depending on the intended application. Filtration processes should be validated. Triple filtration using filters of pore size 0.1 µm has been shown to result
in a high degree of mycoplasma removal. Although filtration may remove some large viruses and viral aggregates from the serum, generally viruses cannot be completely eliminated in this manner. Furthermore, the filters are not known to eliminate the causative agent of bovine spongiform encephalopathy (BSE). Following filtration, serum manufacturers fill filtered serum into sterile containers by aseptic processing in a suitably controlled environment. Serum manufacturers should describe each filtration process step and should perform serum filtration, filling, and sample collection activities following the manufacturer's approved procedures.

IRRADIATION

Serum treatment by gamma irradiation is very common and one of the most effective methods of virus inactivation. The most frequently used minimum dose is 25 kilograys (kGy). Some countries specify higher dose requirements (>30 kGy) for imported serum. Gamma irradiation may inactivate viruses, mycoplasma, and bacteria, but serum end users should ensure that the gamma irradiation process does not negatively affect their specific applications. Irradiation may have adverse effects on serum quality, and these adverse effects tend to increase with higher doses.

Validation of gamma irradiation has two aspects: (1) dose delivery in a defined load configuration and (2) inactivation capacity. Critical irradiation process parameters include product (serum) temperature, packaging size and configuration, dosimeter distribution, and defined minimum/maximum dose received. Dosimeters should be used to monitor the established high-dose and low-dose positions in each irradiation run. If the serum manufacturer makes inactivation claims, these should be supported by the manufacturer's own well-designed viral inactivation studies.

ULTRAVIOLET (UV) TREATMENT

Serum manufacturers may use UV treatment to inactivate viruses, mycoplasma, and bacteria, but manufacturers must validate the process to demonstrate its efficacy. In addition, manufacturers must be aware that UV treatment may have an adverse effect on serum quality and accordingly should consider the effects of UV treatment for each application, as should serum end users.

HEAT INACTIVATION

Heat inactivation involves elevating the temperature of the serum to >56° for at least 30 minutes to inactivate complement. Heat inactivation may also inactivate viruses, mycoplasma, and bacteria; but it may have an adverse affect on serum quality, and manufacturers must validate the procedure’s suitability for specific applications. Heat inactivation provides significantly less assurance of virus inactivation than does irradiation.

VIRAL CLEARANCE STUDIES

Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin (1050) and other regulatory documents give guidance about conducting viral clearance studies that help validate removal/inactivation processes. Serum manufacturers should also perform formal spiking studies with relevant and representative (model) viruses, and should test and compare inactivated spiked serum samples, negative controls, and positive controls.

CHARCOAL STRIPPING

Some serum manufacturers use charcoal/dextran treatment to reduce the levels of hormones in serum.

DIALYSIS

Some manufacturers use dialysis or diafiltration to remove low molecular weight components from serum.

CLEANING AND STERILITY OF EQUIPMENT

Stainless steel systems and tubing used in the manufacture of bovine serum must be cleaned and sterilized to prevent cross-contamination and growth of adventitious agents. Serum manufacturers must validate their cleaning processes for removing and inactivating agents of concern. Thereafter, manufacturers should implement process controls that routinely verify cleaning cycles. Steam sterilization-in-place is a common and effective sterilization technique. Serum manufacturers that use this technology must validate steam cycles to demonstrate their uniformity and ability to destroy heat-resistant bacterial spores. Alternatively, manufacturers can use sterile disposable systems that do not require cleaning validation.

Quality Control

TRACEABILITY

Abattoir Collection: Materials collected in the U.S. should originate from U.S. Department of Agriculture (USDA)-registered facilities. Serum manufacturers should maintain documentation that traces a given serum sub-lot to the abattoir where it was collected. Slaughterhouses maintain records of animal source. General industry practice is to keep this information as part of the Device Master Record. General record-keeping requirements at USDA-licensed abattoir facilities are outlined in 9 Code of Federal Regulations (CFR) 320.

Materials collected from countries approved by the USDA for importation of bovine products into the U.S. should meet the requirements of the competent authority of the country of origin. In addition, serum manufacturers should keep USDA-required safety testing records of imported materials (if applicable) as part of their Device History Record.
Serum manufacturers should consult 9 CFR 309 and 9 CFR 310 about requirements for inspection of animals for various diseases pre- and post-slaughter. These requirements are recommended for materials collected outside the U.S.

**Donor Herd Collection:** Serum manufacturers should maintain traceability to the donor animal farm where blood was collected from donor animals. In most cases, manufacturers individually identify farm animals and keep records for bleed and processing dates, making it possible to trace blood collection to an individual animal. A licensed veterinarian or a designee under the guidance of a veterinarian should inspect animals regularly and should certify that the animals are free of disease and fit for human consumption, consistent with 9 CFR 309.

**PRODUCT STORAGE AND STABILITY**

Serum should be stored in the frozen state at −10° or below. Serum is frozen as quickly as possible to preserve product quality and is stored under controlled storage conditions. Serum manufacturers should establish serum product stability in support of a proposed expiration date. Typical expiration dating for bovine serum is 5 years from the date of filtration and filling. Use of any type of bovine serum beyond the stated expiration date depends on the application, and the serum user must establish the product’s continued suitability for use.

**Labeling**

Finished product labels must contain the following information: product description, lot number, storage conditions, name and address of manufacturer, and a statement indicating the intended use. Materials intended for research purposes are exempt from labeling regulations (21 CFR 801). Typically, serum manufacturers supply a lot-specific Certificate of Analysis (COA) that is classified as part of the product’s labeling. See COA requirements in the following section.

**Certification/Documentation**

**CERTIFICATE OF ANALYSIS**

The COA should provide information about a specific lot of serum, including tests performed and test results (according to the serum manufacturer’s specifications for release), as well as critical labeling identifiers such as lot number, catalog number, description of type of bovine serum, country of origin, and either or both dates of manufacture and expiration. This document is distinct from the certificate of health issued by the competent authority of the country of origin.

**CERTIFICATE OF ORIGIN AND CERTIFICATION OF ANIMAL STATE OF HEALTH**

The Certificate of Origin establishes the country in which the bovine blood was collected and veterinary certification of the health of the animals pre- and postcollection (9 CFR 327.4).

**IMPORT/EXPORT DOCUMENTS**

Import/export documents contain formal certification of animal disease status of the country of origin and negotiated/agreed certification statements. These vary from country to country. Each country defines import/export requirements in order to control introduction of exotic animal diseases and their economic impact as well as product safety assessments (risk vs. research, diagnostic, and/or therapeutic benefits).

**PRODUCTION REPORTS**

Production reports typically are batch records that document the raw materials in identifiable and traceable ways, production methods (centrifugation or filtration) used in manufacturing, equipment and facility cleaning, quality control testing, and personnel performing required activities. Raw material with Certificates of Origin or serum production records facilitates traceability to the source of the blood that was used to create the serum. When serum is used as a raw material for further manufacturing, process documentation also helps demonstrate controlled manufacture of the bovine serum.

**BSE RISK ASSESSMENT**

Despite the low risk potential of transmissible spongiform encephalopathies (TSEs) in bovine serum, various U.S. and international regulatory agencies have developed guidance to help manage and further reduce the potential risks of transmission. In the absence of appropriate test methods of detecting the infectious agent in fluids such as blood, the consensus recommendation from various regulatory agencies is to adopt good risk assessment strategies. This section of the chapter provides some background information on the disease and current methods of detection; it also highlights risk assessment and risk reduction strategies to potentially prevent transmission of the disease through the use of serum in the manufacturing of medicinal products.

**Description of the Disease**

TSEs are transmissible animal and human diseases that are characterized by degeneration of the brain, associated with severe neurological signs and symptoms. Since the outbreaks of TSE in cattle, termed BSE, which were transmitted to other species, public health officials have been concerned about the risk of TSE infection, including the possibility of TSE transmission by the
use of therapeutic products manufactured using bovine serum. In cattle infected with BSE, lower titers have been found in the
cerebrospinal fluid, lung, lymph tissue, spleen, kidney, liver, and ileum. Studies have shown that transfusion of blood from sheep
infected with either BSE or scrapie but without evident disease can infect naïve sheep. Although the risk of cross-contamination
is always present, to date no studies have shown that blood can transmit disease from cattle with BSE. Embryos from BSE-affected
cattle have not transmitted diseases to mice. Calves born of dams that received embryos from BSE-affected cattle have survived
for up to 7 years, and examination of the brains of both the unaffected dams and their offspring revealed no spongiform
encephalopathy.

Detection Strategies

No currently available procedures have been validated as being sufficiently sensitive for routine antemortem screening of
asymptomatic animals, although analytical methods are under development for detection and quantitation from low-infectivity
materials such as blood. The classic diagnostic test for TSEs is postmortem histological examination of brain tissue to confirm
characteristic vacuolar degeneration. Other testing options include immunohistochemical tests that can confirm the presence
of PrPSc, the abnormal disease-specific conformation of prion-related protein (PrP), in the vacuolated regions of the brain; and
immunochemical tests such as Western blots and enzyme-linked immunosorbent assays that can detect PrPSc in tissues with
high or moderately high titers. These tests typically take less time to perform than histological examination (6–8 hours vs. weeks,
respectively) and can be partially or fully automated. Although most of these are postmortem tests, studies have demonstrated
the feasibility of antemortem testing of lymphoid tissue samples from the tonsils or from the third eyelid of infected animals.
Immunochromatographic tests require extensive sample collection and preparation and can be cost prohibitive for routine testing
and monitoring the disease state of large herds. Diagnostic strategies must consider the sensitivity of testing in certain tissues as
well as the test’s ability to detect infectivity in animals before the development of clinical signs of disease. Negative results do
not ensure the absence of infectivity. Detection of infectivity is possible if suspect tissue is inoculated into experimental animals
intracranially where the causative agent can amplify. This approach for detection of low infectivity can take months to years to
yield a positive result.

Risk Assessment and Risk Reduction Strategies

Serum manufacturers should employ risk reduction strategies to eliminate the danger of cross-contamination of fetal blood
with other tissues, including appropriate sourcing of animal-derived articles and using practices that have been shown to
eliminate or minimize the risk of transmitting TSE, via either foods or health care products. Serum end users should perform a
risk assessment of their sourcing strategy that takes into account the amount of bovine serum used in their application and
should conduct supplier audits to ensure traceability of sourcing, handling, and appropriate quality control systems.

SOURCE AND AGE OF ANIMALS

Serum manufacturers should monitor the traceability of each lot of serum to ensure the qualification of bovine serum, as
described previously in the two sections Serum Harvesting and Processing and Quality Control. In addition to traceability, careful
selection of source materials is the most important criterion for the safety of medicinal products. Certification of the origin must
be available from the supplier, and manufacturers should keep this information on file. The U.S. Food and Drug Administration
(FDA) recommendations prohibit the use in FDA-regulated products (except gelatin) of any bovine-derived materials that
originate from countries that report indigenous cases of BSE. The current proposed rule qualifies FBS as an unlikely source of
BSE infectious material, because current evidence suggests that cow-to-calf transmission of BSE is unlikely. The proposed rule
also states that prohibited cattle materials do not include materials sourced from fetal calves of cows that were inspected and
passed, as long as the materials were obtained by procedures that can prevent contamination with specified risk materials. For
veterinary biologics, current regulations enforced by the USDA’s Center for Veterinary Biologics (CVB) indicate that ingredients
of animal origin should be sourced from countries with no or low BSE risk, as defined by the U.S. National Center for Import
and Export and 9 CFR 94.18.

The most satisfactory sources of materials are from countries with the following:
• No reported cases of indigenous BSE
• Compulsory notification of positive tests
• Compulsory clinical and laboratory verification of suspected cases
• Prohibition of the use in ruminant feed of meat and bone meal containing any ruminant protein
• No importation of cattle from countries where a high incidence of BSE has occurred
• No importation of progeny of affected female

BSE infectivity may increase with animal age. Although bovine serum is considered a low-risk material for TSE transmission,
some end users consider it prudent to source serum from dams below a set maximum age. If manufacturers cannot determine
the date of the dam’s birth, they should consider both the implementation date of the feed ban in the country of origin and
the incubation period of BSE in order to determine the safety of the source. A ruminant feed ban was imposed in the United
Kingdom in July of 1988. These considerations are lot specific, so audits of the raw material supplier should include a review of
records.

PRODUCTION PROCESS

End user manufacturing systems should be in place for monitoring the production process and for batch delineation
(definition of batch, separation of batches, and cleaning between batches). Of primary importance is control of the potential
for cross-contamination with possible infectious material. Because of the documented resistance of TSE agents to most
inactivation procedures, controlled sourcing is the most important criterion in achieving acceptable product safety.
Whenever possible, manufacturers should identify steps that theoretically or demonstratively remove or inactivate agents during the manufacture of the material. Manufacturers should continue their investigations into removal and inactivation methods to identify steps/processes that will help ensure the removal or inactivation of TSE agents. Manufacturers should design production processes using available methods that have the greatest likelihood of inactivating or removing TSE agents. For example, prolonged exposure of tissues to high moist heat and high pH inactivates the BSE agent. Such treatments, however, are inappropriate for the extraction of many other types of bovine-derived articles, such as serum, because these treatments lead to the destruction of the material. Conventional chemical and biochemical extraction and isolation procedures may be sufficient to remove the infectious agent. Similar techniques may be effective for other bovine-derived articles. Further research will help to develop an understanding of the most appropriate methodology for validation studies. Issues to consider during validation of a process for removal of TSE agents include the following:

- The nature of the spiked material and its relevance to the natural situation
- Design of the study (including scale-down approaches)
- Method of detecting the agent (in vitro or in vivo assay) after spiking and after the treatment
- Characterization and standardization of reference materials for spiking
- Data treatment and analysis (see Design and Analysis of Biological Assays (11))

Because no studies have successfully validated analytical methods for the detection of small amounts of the TSE agent, TSE clearance validation studies typically employ the intracranial injection of in-process material into rodents for amplification and detection of potential residual infectivity.

## TESTING AND CONTROL OF ADVENTITIOUS AGENTS

### Introduction

Rigorous testing procedures, strict processing and production guidelines, and appropriate risk assessments help ensure the safety of the different types of bovine serum. This section discusses specific tests that can detect and control adventitious agents.

### Adventitious Agents Testing

The adventitious agents testing required for the evaluation of master seeds, master cells, and bulk and final products is described in 9 CFR 113.53 and by directives from the European Agency for the Evaluation of Medicinal Products (EMEA) (EMEA/CVMP/743/00 and EMEA/CPMP/BWP/1793/02). The testing methods outlined in these documents can detect a wide range of bovine microbial agents in serum products. These testing methods meet the requirements for most of the world’s regulatory agencies. Serum manufacturers should test a representative sample of each batch of serum to determine the presence of adventitious agents. Testing is performed after filtration but before any further processing that is intended to inactivate or remove viruses.

Filtration with 100-nm (0.1-µm) pore size filters is an accepted method for removing mycoplasmas and gamma irradiation (> 25 kGy while frozen), and chemical treatments (e.g., with betapropiolactone) are accepted methods of inactivating viruses and mycoplasmas; serum manufacturers routinely use these tools in both production and testing facilities. These treatments do not remove antibodies that may interfere with some applications. Additionally, the treatments do not ensure complete viral removal or inactivation, but can significantly reduce the risk of viral activity. The testing series to screen bovine serum for the absence of adventitious agents typically includes the following:

- Bacterial and fungal sterility testing as described in 9 CFR 113.26
- Mycoplasma testing as described in 9 CFR 113.28
- Viral testing as described in 9 CFR 113.53

The procedures described in Sterility Tests (71) confirm the absence of bacterial and fungal infection. For viruses, only cultivation using suitable substrate cells can indicate viral infectivity and replication. Those who use serum for research or production should test the serum for the absence of adventitious agents in a manner that is consistent with the product’s intended application, bearing in mind that testing indicates only presence or absence of adventitious agents within the limits of the test procedures used.

### Mycoplasma Testing

Mycoplasma contamination in tissue culture can arise from many animal origin sources, including serum, but more commonly it results from cross-contamination of infected cultures. Mycoplasmas are particularly insidious contaminants in cell culture because they

- cannot be visualized by light microscopy even at high density (>10^7 colony-forming units/mL);
- cause no observable change in turbidity or pH of the culture fluid;
- cannot routinely be removed by single sterilizing filters, although removal can be obtained through a triple series of 0.1-µm filters;
- are unaffected by traditional antibiotics used in cell culture; and
- exert an extremely wide variety of adverse effects in tissue culture.

Classical mycoplasma detection is described in Mycoplasma Tests (63).

In addition to these methods, more recent detection procedures include luminescent and polymerase chain reaction (PCR) assay procedures. Nucleic Acid-Based Techniques—Amplification (1127) describes the general principles of PCR assays. The sensitive 20-minute luminescent assay measures a specific enzyme activity of mollicutes that converts adenosine diphosphate
Viral Testing

The virus testing procedures for serum products are outlined in 9 CFR 113.52 and 9 CFR 113.53. In addition, there are other documents that may include equivalent or relevant testing such as EMEA/CPMP/743/00-Rev.2 from the Committee for Veterinary Medicinal Products (CVMP) Revised Guideline on Requirements and Controls Applied to Bovine Serum Used in the Production of Immunological Veterinary Medicinal Products and EMEA/CPMP/BWP/1793/02 from the Committee for Proprietary Medicinal Products (CPMP) Note for Guidance on the Use of Bovine Serum in the Manufacture of Human Biological Medicinal Products. Serum manufacturers should perform virus testing in compliance with this regulation, using at least two different and sensitive detector cell lines, one of which should be of bovine origin. The tests include cultivation of detector cells in cell culture media supplemented with 15% test serum for at least 21 days. Cells are subcultured at least twice during this period, usually 7 and 14 days post inoculation. At the conclusion of the last subculture (after a total of at least 21 days of incubation), cells are examined for general signs of virus amplification. The following end points are used for general virus detection: microscopic cell examination for cytopathogenic agents such as infectious bovine rhinotracheitis virus, cell staining and microscopic examination for inclusion bodies, and hemadsorption test to detect hemadsorbing agents such as PI-3. In addition to this series of testing and at the conclusion of the last subculture (after a total of at least 21 days of incubation), cells are stained with specific fluorescent antibodies against the following specific viral agents:

- BVDV
- Bovine parvovirus
- Bovine adenovirus
- Bluetongue virus
- Bovine respiratory syncytial virus
- Reovirus
- Rabies virus

In addition to the viruses listed above, other viruses can be causative agents of disease and may require testing in various bovine serum applications. The serum end user is responsible for determining whether full 9 CFR testing is sufficient, and if other specific viral agents should be tested for. Examples of specific viruses not covered by the current virus testing guide may include akabane, bovine herpesvirus 1 (BHV-1), Parainfluenza-3 virus (PI-3), bovine leukemia, bovine rotavirus, bovine circovirus, bovine polyomavirus, coronavirus, torovirus, bovine enterovirus, bovine astrovirus, foot-and-mouth disease virus (FMDV), and rinderpest. Appendix 2 provides a general description of some of these viruses as well as the ones for which testing is required. A serum end user’s thorough risk analysis should determine the scope of testing and serum treatment options.

Risk Assessment and Detection Strategies

Serum manufacturers and serum end users should carry out a comprehensive, science-based risk assessment (e.g., Failure Modes and Effects Analysis) in order to better understand the safety profile of the serum product. The following risk assessment elements can be taken into consideration, but other elements can be included as appropriate: country of origin, region of the country, animal disease status of the country/region of origin, animal age, blood collection process, animal stunning method and exsanguination method, serum manufacturing process, type of production quality system, production in-process controls, final product testing, virus inactivation, equipment segregation, equipment cleaning procedure, personnel training, serum use/application, pharmaceutical product type, and intended use.

The species barrier provides a degree of protection against infection by some animal etiologic agents. This barrier is not an alternative to proactively ensuring that pharmaceutical products are manufactured only from raw materials of animal origin that have undetectable levels of adventitious agents. Inoculation of viable organisms into a nonhost species carries a risk that the organisms could cross the species barrier. An appropriate test regimen of serum material should therefore include examination for potential contaminants associated with the species of origin and the species of intent. Serum treatments to inactivate viral agents are a factor in establishing the appropriate test regimen for a particular material. Lowest risk of contamination is associated with biological materials that are terminally sterilized.

Zero risk is neither possible nor reasonable. The serum manufacturers should fully describe specific testing regimens in the product specifications, and these will vary depending on the type and source of the serum. Therefore, the guidelines for screening described in this chapter are examples only, and screening for all viruses listed may not be required for a particular material. Some manufacturers may perform certain tests on the finished product or on in-process materials rather than on individual component(s). Manufacturers must also evaluate the dilution effect in relation to the limit of detection of the test procedure. Interference with growth or neutralization of viral activity by serum may be an indication of a specific antibody or certain nonspecific factors in serum masking the viral agent. It is recommended that serum manufacturers consider this possibility when determining an adequate level of treatment in their viral inactivation studies or in virus testing applications.

Serum manufacturers should confirm that the species of origin is bovine to ensure that no other nonbovine agents may be present. Manufacturers should perform extraneous virus testing in appropriate cell cultures (see Virology Test Methods (1237) for appropriate cell line choices dependent on assay and targeted agent). If necessary, seroconversion studies should be conducted in susceptible animal species using a host species immune antibody response as the method of detection. Studies should use this procedure following an inactivation step to detect whether the virus was present before the virus inactivation process.

Serum manufacturing processes should be conducted in a consistent manner, following the established manufacturing procedures, with adequate quality systems built into the production process. Furthermore, equipment segregation (by species
of origin), equipment and facility cleaning procedures, and personnel training are important elements in the risk assessment of the process.

**Safety Considerations**

End users of donor bovine serum may require serum that does not have detectable antibodies against BVDV or other specific agents so that the users can propagate cell cultures used in vaccine production, diagnostic testing, and test kit preparation, especially for the maintenance of master seed and master cell stocks. More than 40 cell types are available for the production of veterinary biologicals, but fewer than 10 media types are available for their propagation. Some researchers have proposed serum-free media as an alternative in propagating certain cells and viruses; but this means adapting culture procedures, which may alter the cells and change production results. If new or different sera are imported into the U.S., serum end users will require confirmation of source, species, and documentation of the origin of the sera in countries that are free of FMD and rinderpest.

**CHARACTERIZATION OF BOVINE SERUM**

**Introduction**

In the absence of end product-specific requirements, each lot of FBS should be tested to confirm that the serum meets the requirements of the proposed general chapter *Fetal Bovine Serum—Quality Attributes and Functionality Tests* (90). For all other types of bovine sera, this section describes several key procedures for characterization. These procedures are not mandatory but are guidelines that manufacturers may consider for their individual applications. The table in the *Hemoglobin* section shows samples of specifications for the different types of bovine sera.

**Species Identification**

Both inter- and intraspecies identification assays should be performed on bovine sera to confirm species identity and the integrity of the serum products, and to ensure that nonbovine agents are not present. The most commonly used assay for the identification of bovine species identity is based on the electrophoretic profile of specific serum proteins. With electrophoresis, the serum proteins usually separate into as many as six fractions: albumin, alpha 1, alpha 2, beta 1, beta 2, and gamma globulins.

Other procedures used for bovine speciation include radial immunodiffusion (RID) and the double diffusion Ouchterlony method. These procedures allow either qualitative or quantitative measurements of the immunoglobulin G levels in serum. The RID method is based on the diffusion of an antigen from a circular well into a homogeneous gel that contains specific antisera for each particular antigen. A circle of precipitated antigen and antibody forms and continues to grow until it reaches equilibrium. The diameters of the rings are a function of antigen concentration. The Ouchterlony method is a double gel diffusion test wherein antigen and antibody diffuse toward each other in a semisolid medium to a point in the medium where optimum concentration of each is reached, forming a precipitate. The Ouchterlony plates contain cylindrical wells—a central 8-mm diameter antigen well, surrounded by six 3-mm antisera wells—which make possible the simultaneous monitoring of multiple antigen–antibody systems and the identification of particular antigens in a preparation. The proposed general chapter *Fetal Bovine Serum—Quality Attributes and Functionality Tests* (90) describes the accepted procedure.

**Hemoglobin**

Hemoglobin is a multi-subunit protein that forms an unstable reversible bond with oxygen in the red blood cells. The oxygen-loaded form is called oxyhemoglobin and is bright red. The oxygen-unloaded form is called deoxyhemoglobin and is purple-blue. Oxyhemoglobin is the predominant form in red blood cells.

Low hemoglobin content in sera is widely accepted as a good general indication of rapid and careful processing of blood that will be used for serum. Red blood cells are fragile and rupture easily, releasing hemoglobin into the serum. Rough handling of the harvested blood, poor temperature control, or delayed processing elevates hemoglobin content in serum. Acceptable levels of hemoglobin may vary with intended application. The hemoglobin levels are measured using spectrophotometric procedures (see *Ultraviolet-Visible Spectroscopy* (857)) as described in the proposed general chapter *Fetal Bovine Serum—Quality Attributes and Functionality Tests* (90).

<table>
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<tr>
<th></th>
<th>FBS</th>
<th>Newborn Calf Serum</th>
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<tr>
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<td>7.00–8.00</td>
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</table>

Published on March 26, 2020
Chemical Profile

The testing of components such as cholesterol, alpha globulin, beta globulin, gamma globulin, albumin, creatinine, bilirubin, glucose, alanine aminotransferase, aspartate aminotransferase, phosphorus, potassium, calcium, and sodium usually is not considered a criterion for bovine serum lot release. Some manufacturers do not perform the tests on a routine basis but only as auxiliary tests. In some instances hospital clinical laboratories may run the tests. The levels of these chemicals in serum are important to end users and may also be used to assess lot-to-lot variability.

Endotoxin Levels

Although high endotoxin levels are not suitable for applications involving injectables, acceptable levels in bovine sera vary depending on the intended application. Some manufacturers may overlook the importance of low endotoxin levels in bovine sera used in cell culture applications. Endotoxin influences more than 30 biological activities. Some of these are macrophage activation, mitogenic stimulation, and induction of interferon and colony-stimulating factor (for some applications, these may be positive activities). Endotoxin can also lead to cytotoxicity by initiating complement activation. The most commonly used methods for endotoxin detection are the semiquantitative gel clot Limulus amebocyte lysate procedure and the quantitative kinetic chromogenic method described in Bacterial Endotoxins Test (85). For both the gel clot and the kinetic chromogenic assays, valid endotoxin assays require appropriate treatment by heat or dilution in order to avoid adverse effects of interfering substances in serum. Researchers should include a positive product control in each assay to confirm that any interference has been overcome by the heat or dilution treatment.

Osmolality

The osmolality test is designed to evaluate the electrolyte concentration in bovine serum. Chemicals that affect serum osmolality include sodium, chloride, bicarbonate, potassium, proteins, and glucose. Serum manufacturers should measure the osmolality of each serum batch to verify compliance with product specifications, using equipment calibrated with standards that are traceable to the National Institute of Standards and Technology. Osmolality (785) describes how osmolality is determined by freezing-point depression of the bovine serum solution. Scientists use at least two standards to calibrate the instrument. The osmolality of each sample is calculated and related to the serum water content and is expressed as mOsmol/kg H$_2$O.

Total Protein Level

The total protein level in serum is measured to verify animal age and compliance with product specifications. Biotechnology-Derived Articles—Total Protein Assay (1057) describes two procedures, the Biuret and Bradford methods, for determining protein concentration. The acceptable level of protein in serum should be assessed by the end user based on the intended application.

Cell Growth Properties

Each lot of serum should be tested for its ability to support in vitro growth of specific cell lines. Bovine sera are highly variable, and different lots may yield different results. Because of this variability, end users should characterize and standardize the cell lines that they will use for this type of testing. End users should design cell growth procedures that will help them check the efficacy of bovine serum in promoting cell growth. Serum manufacturers will benefit from monitoring growth promotion over several generations of subcultures to detect any evidence of cytotoxicity or changes in cell morphology. Different serum manufacturers use different cell types, and the growth studies and cell lines used by serum manufacturers also may differ from those applied by serum end users. When serum manufacturers evaluate the growth properties of a specific cell line in response to a specific lot of serum, they should take into account plating efficiency and/or growth promotion or some other functionality tests that qualify the serum lot for its intended use.

Plating efficiency at low cell density is a preferred method for analyzing the proliferative capacity and survival of single cells under optimal growth conditions. This procedure can reveal differences in the growth rate within the population and is capable of distinguishing between changes in growth rate (colony size) and cell survival (colony number). The growth kinetic is another important aspect in the design of cell-based experiments. Determining the growth curve of each cell line helps define optimal culture conditions, because variation in serum and other growth additives may influence growth parameters, which may affect the experimental outcome.

In the absence of specific tests designed for their particular products, serum users can refer to the functionality tests described in the proposed general chapter Fetal Bovine Serum—Quality Attributes and Functionality Tests (90) to determine whether a lot of serum is suitable for their application. This chapter provides guidance about how to perform growth promotion and plating efficiency tests.

In Vitro Cytotoxicity

Serum manufacturers should use an appropriate cell line for testing each lot of serum, and should perform growth studies through several subcultured generations to ensure that the serum has no cytotoxic effect on the cells. The choice of cell line depends on the intended use of serum. The cell growth and cytotoxicity assays should be performed on the final batch of serum after any viral inactivation step or any further processing.
**CONCLUSION**

Bovine serum is likely to remain an important component in the manufacture of many biologics, particularly those relying on cell culture. As with similar materials, bovine serum displays inherently variable quality. As a result, serum end users must establish suitable tests, procedures, and acceptance criteria for introduction of materials into a particular application process that uses serum. This may mean screening multiple lots of bovine serum to determine which lots meet the specification (see the section *Characterization of Bovine Serum*).

Manufacturers of therapeutic products using bovine serum are responsible for ensuring and documenting its quality and its impact on the quality, safety, and efficacy of the final product. In addition, it is important to ensure that each lot of serum performs in an equivalent manner during manufacturing. Serum can also interfere with final product purification; therefore it is important to understand the effect of bovine serum on the manufacturing process in order to understand the effect that various processes might have on the final product. Finally, risks can also be mitigated through the design of processes to include steps to adequately remove the bovine material through dilution, separation, or inactivation as well as the development of analytical assays to assess the bovine-derived residual content during processes and in the final therapeutic product. A number of sensitive assays can provide a quantitative means of detecting bovine material at picogram levels.

**APPENDICES**

**Appendix 1: Relevant Regulatory References**

Bovine sera and serum-related products used in the manufacture of biological products are regulated in the context of Requirements for Ingredients of Animal Origin Used for the Production of Biologics, 9 CFR 113.53. Currently, individual serum manufacturers perform detection studies to identify contaminating viruses. Because of the potential international market for serum, serum manufacturers need to be mindful of other regulatory requirements. Manufacturers can use the documents listed here as guidance for screening bovine sera for contamination by adventitious agents. Because of the risk carried by animal-derived serum products, serum manufacturers and end users should ensure that the country of origin of the material complies with applicable regulatory requirements. Although no cell performance assays currently demonstrate lack of BSE in serum, serum manufacturers must comply with the regulatory requirements of countries where the serum is sourced and marketed to ensure a minimal risk of infection with BSE/TSE.

Beyond relevant *USP* chapters referenced in this chapter, the following list of documents includes regulatory documents as well as best practices in product and process development, manufacturing, quality control, and quality assurance.

**CFR**
- 9 CFR 94.18 (CVB, 2001)
- 9 CFR 113.46
- 9 CFR 113.47
- 9 CFR 113.52
- 9 CFR 113.53
- 9 CFR 113.55
- 9 CFR 320
- 9 CFR 327.4
- 21 CFR 211 Subpart E
- 21 CFR 801.1
- 21 CFR 809.10

**FDA**
Appendix 2: Viruses to Consider when testing Bovine Serum

Following is a general description of viruses that manufacturers can consider when testing bovine serum for the absence of adventitious agents. The list is intended only to provide general information. The list of required testing is described in this chapter in the section Viral Testing.

AKABANE

An insect-transmitted virus that causes congenital abnormalities of the central nervous system in ruminants. Disease due to Akabane virus has been recognized in Australia, Israel, Japan, and Korea. Antibodies to it have been found in a number of countries in Southeast Asia, the Middle East, and Africa. The disease affects fetuses of cattle, sheep, and goats. Asymptomatic infection has been demonstrated serologically in horses, buffalo, and deer (but not in humans or pigs) in endemic areas.

BLUETONGUE

An infectious, noncontagious arthropod-borne viral disease primarily of domestic and wild ruminants. Infection with bluetongue virus is common worldwide but is usually subclinical or mild. Bluetongue virus is the type-species of the genus Orbivirus in the family Reoviridae. Worldwide, 24 serotypes have been identified, although not all serotypes exist in any one geographic area: e.g., only 5 serotypes (2, 10, 11, 13, and 17) have been reported in the U.S. Distribution throughout the world parallels the spatial and temporal distribution of vector species of Culicoides biting midges, which are the only significant natural transmitters of the virus.

BOVINE ADENOVIRUS

Associated with a wide spectrum of diseases. Bovine adenovirus type 3 is the serotype most often associated with bovine respiratory disease. Bovine adenoviruses are DNA viruses that have been separated into two genera: the Mastadenovirus, or mammalian adenoviruses, and the Aviadenovirus, or avian adenoviruses. Within the genus Mastadenovirus are numerous species-specific serotypes, nine of which have been identified in cattle. Epitheliotrophic adenoviruses have also been isolated from ruminants, and usually are clinically unapparent. Clinical disease is dictated by various factors, including the strain of virus, concurrent infection, stress, environmental conditions, and management practices.

BOVINE HERPESVIRUS 1 (BHV-1)

Associated with several diseases in cattle, including infectious bovine rhinotracheitis, infectious pustular vulvovaginitis, balanoposthitis, conjunctivitis, abortion, encephalomyelitis, and mastitis. BHV-1 infections are widespread in the cattle population. In feedlot cattle the respiratory form is most common.

BOVINE LEUKEMIA

An exogenous C-type oncovirus in the family Retroviridae. Bovine leukemia is a viral disease of adult cattle characterized by neoplasia of lymphocytes and lymph nodes. Infection occurs by iatrogenic transfer of infected lymphocytes and is followed by a permanent antibody response. The prevalence of infection in a herd may be high, but only a few animals develop fatal lymphosarcoma. Infection is spread by contact with contaminated blood from an infected animal.

BOVINE REOVIRUS

Double-stranded ribonucleic acid (RNA) (dsRNA) viruses with nonenveloped spherical virions 60–80 nm in diameter. They cause bovine respiratory diseases.

BOVINE RESPIRATORY SYNCYTIAL VIRUS (BRSV)

An RNA virus classified as a pneumovirus in the Paramyxovirus family. This virus was named for its characteristic cytopathic effect—the formation of syncytial cells. In addition to cattle, sheep and goats can also be infected by respiratory syncytial viruses. Human respiratory syncytial virus (HRSV) is an important respiratory pathogen in infants and young children. HRSV has antigenic subtypes, and preliminary evidence suggests the existence of antigenic subtypes of BRSV. BRSV is distributed worldwide, and the virus is indigenous in the cattle population. BRSV infections associated with respiratory disease occur predominantly in young beef and dairy cattle.

BOVINE ROTAVIRUS

A dsRNA spherical virion 60–80 nm in diameter without an envelope. It is the most common viral cause of diarrhea in calves and lambs.
BOVINE VIRAL DIARRHEA VIRUS (BVDV)

An RNA virus classified as a *Pestivirus* in the family Flaviviridae. BVDV can cross the placenta and appears to be capable of inducing immunosuppression, which allows the development of secondary bacterial pneumonia. BVDV has been reported to be the virus most frequently associated with multiple viral infections of the respiratory tract of calves.

FOOT-AND-MOUTH DISEASE (FMD)

A highly infectious viral disease of cattle, pigs, sheep, goats, buffalo, and artiodactyl wildlife species. In a susceptible population, morbidity approaches 100%. The disease is rarely fatal except in young animals. FMD is caused by an *Aphthovirus* of the family Picornaviridae. Seven immunologically distinct serotypes are known, and within each serotype exist a large number of strains that exhibit a spectrum of antigenic characteristics.

PARAINFLUENZA-3 VIRUS (PI-3)

An RNA virus classified in the Paramyxovirus family. Although PI-3 is capable of causing disease, the virus usually is associated with mild to subclinical infection. The most important role of PI-3 is to serve as an initiator that can lead to the development of secondary bacterial pneumonia. Infections caused by PI-3 are common in cattle.

PARVOVIRUS

A relatively heat-stable single-stranded DNA virus approximately 20 nm in diameter that has been recovered from cattle but under natural conditions is not known to cause disease.

Rabies

An acute viral encephalomyelitis that principally affects carnivores and bats, although it can affect any mammal. Rabies is caused by *Lyssaviruses* in the Rhabdovirus family. Although they are usually confined to one major reservoir species in a given geographic area, spillover to other species is common.

Rinderpest

A *Morbillivirus*, closely related to the viruses that cause canine distemper and measles. Strains may vary markedly in host range and virulence. Sera from recovered or vaccinated cattle cross-react with all strains in neutralization tests, but minor antigenic differences have been demonstrated. The virus is fragile and becomes rapidly inactivated by heat and light but remains viable for long periods in chilled or frozen tissues. Rinderpest is endemic in many countries in Asia and Africa. Historically, the virus has been widely distributed throughout Europe and Africa but to date has not established itself in North America, Central America, the Caribbean Islands, South America, Australia, or New Zealand. Rinderpest is included in the WHO’s Office International des Epizooties list of communicable diseases that have the potential for very serious and rapid spread, irrespective of national borders; that are of serious socioeconomic or public health consequence; and that are of major importance in the international trade of livestock and livestock products.

**〈1030〉 BIOLOGICAL ASSAY CHAPTERS—OVERVIEW AND GLOSSARY**

The suite of USP bioassay chapters focuses on relative potency assays. These assays recognize the inherent variability in biological test systems (whether animals or cells) that may be seen from laboratory to laboratory and from day to day. That inherent variability compromises the reliability of an absolute measure of potency. In relative potency assays, the biological activity of a Test material is compared to the activity of a Standard in an assay system wherein the use of a Standard reduces the influence of the inherent variability of the system on the estimation of relative potency. Relative potency assays also provide focus on important variability in response because of differences between the Test and Standard materials (if such a difference exists). The Test is expected to behave as a dilution or concentration of the Standard and should exhibit the property of similarity. Although they are intended for relative potency bioassays, the principles and practices developed in these chapters may have wider application—for example, to immunoassays and receptor-ligand–binding assays used to determine relative potency.

Chapter 〈1032〉 provides information for scientists developing a new biological assay. As seen in Table 1, the chapter covers a range of activities across the life cycle of the assay, with emphasis on development leading to validation, including the choice of test system and design considerations (e.g., plate layout). It also addresses data analysis strategies that should be considered during development (before validation) but that are not routinely addressed later. Among these strategies are the choice of weighting scheme, data transformation, if any, and choice of statistical model. Statistical details in support of these sections of chapter 〈1032〉 are found in chapter 〈1034〉.
Table 1. Primary Sections of Design and Development of Biological Assays (1032)

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Chapter (1034) provides information about the data analyses appropriate for common relative potency bioassays, including parallel-line, slope-ratio, parallel-curve, and quantal models. The chapter also includes analyses supporting system and sample suitability assessment and methods for combining results from independent assays (see Table 2). This chapter is the most statistically advanced of the three chapters but is designed to be suitable for both biologists and statisticians. The conceptual material requires only a minimal statistical background. Methods sections require a statistical background at the level of Analytical Data—Interpretation and Treatment (1010) and familiarity with linear regression.

Table 2. Primary Sections of Analysis of Biological Assays (1034)

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Table 2. Primary Sections of Analysis of Biological Assays (1034) (continued)

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Chapter (1033) is intended to follow chapters (1032) (assay development) and (1034) (development of data analysis plans). That is, chapter (1033) assumes a fully developed bioassay (including a data analysis plan and at least tentative values for system and sample suitability criteria and the bioassay format) and provides guidance about the validation of that assay. The chapter addresses the validation characteristics relevant to relative potency bioassays and provides more detail regarding the statistical methods used in validation than does Validation of Compendial Procedures (1225). Principles and practices developed in chapter (1033), although they are intended for relative potency assays, may have wider application. The chapter emphasizes validation approaches that provide flexibility in adopting new bioassay methods, new biological drug products, or both (see Table 3).

Table 3. Primary Sections of Biological Assay Validation (1033)

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GLOSSARY

This glossary pertains to biological assays and provides a compendial perspective that is consistent across USP–NF’s suite of bioassay chapters, is complementary to previous authoritative usage, and provides a useful focus on the bioassay context. In many cases the terms cited here have common, though undocumented, usages or are defined in Validation of Compendial Procedures (1225) and in the International Conference on Harmonization (ICH) Guideline Q2(R1), Validation of Analytical Procedures: Text and Methodology. (Chapter (1225) and ICH Q2(R1) agree on definitions.) The Glossary is intended to be consistent with these precedent usages, and notes are provided when a difference arises because of the bioassay context.

Definitions from (1225) and ICH Q2(R1) are identified as, for example, “(1225)” if taken without modification, or “adapted from (1225)” if taken with minor modification for application to bioassay. Most definitions are accompanied by notes that elaborate on the bioassay context.

The terms are organized alphabetically within five topic sections:
I. General terms related to bioassays
II. Terms related to performing a bioassay
III. Terms related to precision and accuracy
IV. Terms related to validation
V. Terms related to statistical design and analysis

*Table 4* shows each term and the *Glossary* section in which it can be found.

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I. General Terms Related to Bioassays

**ANALYTICAL PROCEDURE [ADAPTED FROM Q2(R1)]**

Detailed description of the steps necessary to perform the analysis.

[NOTE—1. The procedure may include but is not limited to the sample preparation, the Reference Standard, and the reagents; use of equipment; generation of the standard curve; use of the formulae for the calculation; etc. 2. An FDA Guidance provides a list of information that typically should be included in the description of an analytical procedure.]

**ASSAY**

Analytical procedure to determine the quantity of one or more components or the presence or absence of one or more components.

[NOTE—1. Assay often is used as a verb synonymous with test or evaluate, as in “I will assay the material for impurities.” In this glossary, assay is a noun and is synonymous with analytical procedure (q.v.). 2. The phrase to run the assay means to perform the analytical procedure as specified. 3. In common practice, assay and run (q.v.) often are used interchangeably. In this glossary, they are different. Also see bioassay and bioassay data set.]

**ASSAY DATA SET**

The set of data used to determine a single potency or relative potency for all samples included in the bioassay.

[NOTE—1. The definition of an assay data set can be subject to interpretation as necessarily a minimal set. It may be possible to determine a potency or relative potency from a set of data but not do this well. It is not the intent of this definition to mean that an assay data set is the minimal set of data that can be used to determine a relative potency. In practice, an assay data set should include, at least, sufficient data to assess similarity (q.v.). It also may include sufficient data to assess other assumptions. 2. It is also not an implication of this definition that assay data sets used together in determining a reportable value (q.v.) are necessarily independent from one another, although it may be desirable that they be so. When a run (q.v.) consists of multiple assay data sets, independence of assay sets within the run must be evaluated.]

**BIOASSAY, BIOLOGICAL ASSAY** (these terms are interchangeable)

Analysis (as of a drug) to quantify the biological activity or activities of one or more components by determining its capacity for producing an expected biological activity on a culture of living cells (in vitro) or on test organisms (in vivo), expressed in terms of units.

[NOTE—1. The components of a bioassay include the analytical procedure, the statistical design for collecting data, and the method of statistical analysis that eventually yields the estimated potency or relative potency. 2. Bioassays can be either direct or indirect.]

- **Direct bioassays**—Bioassays that measure the concentration of a substance that is required in order to elicit a specific response. For example, the potency of digoxin can be directly estimated from the concentration required to stop a cat’s heart. In a direct assay, the response must be distinct and unambiguous. The substance must be administered in such a manner that the exact amount (threshold concentration) needed to elicit a response can be readily measured and recorded.
- **Indirect bioassays**—Bioassays that compare the magnitude of responses for nominally equal concentrations of reference and test preparations rather than test and reference concentrations that are required to achieve a specified response. Most biological assays in USP–NF are indirect assays that are based on either quantitative or quantal (yes/no) responses.

**POTENCY [21 CFR 600.3(S)]**

The specific ability or capacity of the product, as indicated by appropriate laboratory tests or by adequately controlled clinical data obtained through the administration of the product in the manner intended, to effect a given result.

[NOTE—1. A wholly impotent sample has no capacity to produce the expected specific response, as a potent sample would. Equi potent samples produce equal responses at equal dosages. Potency typically is measured relative to a Reference Standard or preparation that has been assigned a single unique value (e.g., 100.0) for the assay; see relative potency. At times, additional qualifiers are used to indicate the physical standard employed (e.g., “international units”). 2. Some biological products have]

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multiple uses and multiple assays. For such products there may be different reference lots that do not have consistently ordered responses across a collection of different relevant assays. 3. [21 CFR 610.10] Tests for potency shall consist of either in vitro or in vivo tests, or both, which have been specifically designed for each product so as to indicate its potency in a manner adequate to satisfy the interpretation of potency given by the definition in 21 CFR 600.3(s).]

RELATIVE POTENCY

A measure obtained from the comparison of a Test to a Standard on the basis of capacity to produce the expected potency. [NOTE—1. A frequently invoked perspective is that relative potency is the degree to which the Test preparation is diluted or concentrated relative to the Standard. 2. Relative potency is unitless and is given definition, for any test material, solely in relation to the reference material and the assay.]

REPORTABLE VALUE

The value that will be compared to an acceptance criterion. [NOTE—1. The acceptance criterion for comparison may be in the USP monograph, or it may be set by the company, e.g., for product release. 2. The term reportable value is inextricably linked to the “intended use” of an analytical procedure. Assays are performed on samples in order to yield results that can be used to evaluate some parameter. Assays may have different summary values or formats for different purposes (e.g., lot release vs. calibration of a new reference standard). The reportable value may have different names even if the mechanics of the test itself are identical. Validation is required in order to support the properties of each choice of reportable value. In practice there may be one physical document that is the analytical procedure used for more than one application, but each application must be detailed separately within that document. Alternatively, there may be separate documents for each application. 3. When the inherent variability of a biological response, or that of the log potency, precludes a single assay data set’s attaining a value sufficiently accurate and precise to meet a specification, the assay format may be changed as necessary. The number of blocks or complete replicates needed depends on the assay’s inherent accuracy and precision and on the intended use of the reported value. It is practical to improve the precision of a reported value by reporting the geometric mean potency from multiple assays. The number of assays used is determined by the relationship between the precision required for the intended use and the inherent precision of the assay system.]

RUN

The performance of the analytical procedure that can be expected to have consistent precision and trueness; usually, the assay work that can be accomplished by a single analyst in a set time with a given unique set of assay factors (e.g., standard preparations). [NOTE—1. There is no necessary relationship of run to assay data set (q.v.). The term run is laboratory specific and relates to the laboratory’s physical capability and environment for performing the work of an assay. An example of a run is given by one analyst’s simultaneous assay of several samples in one day’s bench work. During the course of a single run, it may be possible to determine multiple reportable values. Conversely, a single assay data set may include data from multiple runs. 2. From a statistical viewpoint, a run is one realization of the factors associated with intermediate precision (q.v.). Within-run variability is thus repeatability. It is good practice to associate runs with factors that are significant sources of variation in the assay. For example, if cell passage number is an important source of variation in the assay response obtained, then each change in cell passage number initiates a new run. If the variance associated with all factors that could be assigned to runs is negligible, then the influence of runs can be ignored in the analysis, and the analysis can focus on combining independent analysis data sets. 3. When a run contains multiple assays, caution is required regarding the independence of the assay results. Factors that typically are associated with runs and that cause lack of independence include cell preparations, groups of animals, analyst, day, a common preparation of reference material, and analysis with other data from the same run. Even though a strict sense of independence may be violated because some elements are shared among the assay sets within a run, the degree to which independence is compromised may have negligible influence on the reportable values obtained and should be verified and monitored.]

SIMILAR PREPARATIONS

The property that the Test and the Standard contain the same effective constituent, or the same effective constituents in fixed proportions, and all other constituents are without effect in some specific assay context. [NOTE—1. Having similar preparations is often summarized as the property that the Test behaves as a dilution (or concentration) of the Standard. 2. Similar preparations are fundamental to methods for determination of relative potency. Given similar preparations, a relative potency can be calculated, reported, and interpreted. In the absence of similar preparations, a meaningful relative potency cannot be reported or interpreted. 3. The practical consequence of similar preparations is algebraic similarity (q.v.). (Also see Parallelism, section V.)]

SIMILARITY (ALGEBRAIC)

The Test and Standard concentration–response curves are algebraically related in a manner consistent with similar preparations. [NOTE—1. Examples of similarity are parallelism (q.v.) of concentration–response curves and equality of intercepts in slope ratio models. 2. Failure to statistically demonstrate dissimilarity between a Reference and a Test does not amount to demonstration of similarity. To demonstrate similarity an equivalence approach is appropriate; see (1032) and (1034). 3. Similarity is typically a sample suitability (q.v.) criterion. Note, however, that suitability is a necessary but not sufficient condition for preparations to be similar. In practice, absent knowledge of differences between the Test and Standard materials, demonstration of similarity is accepted as demonstrating similar preparations.]
II. Terms Related to Performing a Bioassay

FORMAT, BIOASSAY

The intra- and inter-run replication strategy for replication of assay data sets that has been determined by variance analysis to support the use of the bioassay.

[NOTE—1. Modifications to bioassay format may occur as new information regarding sources of variability becomes available. Such modifications do not include changes to the dilution scheme of Test samples or Standard, or the replication strategy (part of what is sometimes called bioassay configuration). Assay configuration can include nested dimensions like plate design, multiple plates per day, single plates on multiple days, etc. 2. The geometric mean relative potency determined from the bioassay format is the reportable value, which may be used to assess conformance to specifications or as a component of subsequent analysis (e.g., stability evaluation).]

OUT OF SPECIFICATION (OOS)

The property of a reportable value that falls outside its specification acceptance criterion.

[NOTE—Out of specification is not a property of the bioassay but rather a property of Test samples. The term is introduced into (1033) in conjunction with setting validation acceptance criteria which limit the risk of producing out-of-specification test results because of bioassay performance characteristics.]

SAMPLE SUITABILITY

A sample is suitable (may be used in the estimation of potency) if its response curve satisfies limits on critical properties that are stated in the assay procedure.

[NOTE—Response curve properties are to be taken generally; i.e., includes outliers and variability. The most significant of these properties for bioassays is similarity (q.v.) to the standard response curve. In addition, all assay systems have limits on the range of values they can report. For samples that fail one or more sample suitability criteria in a bioassay, the potency estimate from those samples should not be used as a reportable value or as a contributor to a reportable value. Also see truncation bias in this Glossary and the sections Sample Suitability and Range in general chapter (1032).]

SYSTEM SUITABILITY

An assay system is suitable for its intended purpose if it is capable of providing legitimate measurements as defined in the assay protocol.

[NOTE—System suitability may be thought of as an assessment of whether there is any evidence of a problem in the assay system. An example is provided by positive and negative controls, where values outside their normal ranges suggest that the assay system is not working properly.]

III. Terms Related to Precision and Accuracy

ACCURACY (1225)

The closeness of test results obtained by the procedure and the true value.

[NOTE—1. ICH and USP give the same definition of accuracy. However, ISO specifically regards accuracy as having two components, bias and precision.3 That is, to be accurate as used by ISO, a measurement must both be on target (have low bias) and be precise. In contrast, ICH Q2(R1) states that accuracy is sometimes termed “trueness” but does not define trueness. ISO defines trueness as the “closeness of agreement between the average value obtained from a large series of test results and an accepted reference value” and indicates that “trueness is usually expressed in terms of bias.” The 2001 FDA Guidance on Bioanalytical Method Validation4 defines accuracy in terms of “closeness of mean test results obtained by the method to the true value (concentration) of the analyte” (emphasis added) and thus is consistent with the ICH usage. This glossary adopts the USP/ICH approach. That is, accuracy is defined as the agreement between the mean (or expected results) from an assay and the true value, and uses the phrase accurate and precise to indicate low bias (accurate) and low variability (precise). 2. Considerable caution is needed when using or reading the term accuracy. In addition to the inconsistency between USP/ICH and ISO, common usage is not consistent. 3. For purposes of bioassay validation, the terms accuracy and bias have been replaced by relative accuracy and relative bias.]

ERROR, TYPES OF

Two sources of errors that affect the uncertainty of results of a biological assay are systematic error and random error.

A systematic error is one that happens with similar magnitude and consistent direction repeatedly. This introduces a bias in the determination. Effective experimental design, including randomization and/or blocking, can reduce systematic error.

A random error is one whose magnitude and direction vary without pattern. Random error is an inherent variability or uncertainty of the determination. Conversion of systematic into random error, through experimental design or


randomization, increases the robustness of a biological assay and allows a comparatively simple analysis of assay data but may require a larger sample size.

**FORMAT VARIABILITY**

Predicted variability for a particular bioassay format.

**GEOMETRIC COEFFICIENT OF VARIATION**

Found as antilog(\(S\)−1), where \(S\) is the standard deviation determined in the log scale.

[NOTE—The geometric coefficient of variation is usually reported as a percentage (%GCV). It is important not to confuse the %GCV with the %CV. The %GCV is a measure of spread relevant to data analyzed in the log-transformed \([Y = \log(X)]\) scale, and the %CV is a measure relevant to data analyzed in the original \((X)\) scale.]

**GEOMETRIC STANDARD DEVIATION (GSD)**

The variability of the log-transformed values of a lognormal response expressed as a percentage in the untransformed scale. It is found as antilog(\(S\)), where \(S\) is the standard deviation determined in the log scale.

[NOTE—For example, if the standard deviation of log potency is \(S\) using log base 2, the GSD of potency is \(100 \times 2^{S}\).]

**INTERMEDIATE PRECISION (ADAPTED FROM 〈1225〉)**

Within-laboratory precision associated with changes in operating conditions.

[NOTE—1. Factors contributing to intermediate precision involve anything that can change within a given laboratory and that may affect the assay, including different days, different analysts, different equipment, etc. Intermediate precision is thus “intermediate” in scope between the extremes of repeatability (intra-assay) and reproducibility (inter-laboratory). 2. Any statement of intermediate precision should identify the factors that varied. For example, “The intermediate precision associated with changing equipment and operators is...” 3. Investigators can benefit from separately identifying the precision associated with each source (e.g., inter-analyst precision). This may be part of assay development and validation when there is value in identifying the important contributors to intermediate precision. 4. When reporting intermediate precision, particularly for individual sources, care should be taken to distinguish between intermediate precision variance and components of that variance. The intermediate precision variance includes repeatability and thus must be at least as large as the repeatability variance. A variance component, e.g., associated with analyst, also is a part of the intermediate precision variance for analyst, but it could be negligible and need not be larger in magnitude than the repeatability variance.]

**PRECISION (〈1225〉)**

Measure of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of a homogeneous sample.

[NOTE—1. Precision may be considered at three levels: repeatability (q.v.), intermediate precision (q.v.), and reproducibility (q.v.). 2. Precision should be investigated using homogeneous, authentic samples. However, if it is not possible to obtain a homogeneous sample, precision can be investigated using spiked samples that mimic a true sample or a sample solution. 3. Precision may be expressed as the variance, standard deviation, coefficient of variation, or geometric coefficient of variation (q.v.).]

**RELATIVE BIAS**

Measure of difference between the expected (or mean) value and the true value, expressed as a percentage of the true value.

**REPEATABILITY (〈1225〉)**

The precision within a laboratory over a short interval of time, using the same analyst with the same equipment.

[NOTE—1. ICH Q2(R1) says that repeatability is also termed “intra-assay” precision. In the bioassay context, the better term is intra-run, and a “short interval of time” connotes within-run. 2. The idea of a “short interval of time” can be problematic with bioassays. If a run requires multiple weeks and consists of a single assay set, then intra-run precision cannot be determined. Alternatively, if a run consists of two assay data sets and a run can be done in a single day, repeatability of the relative potency determination can be assessed.]

**REPRODUCIBILITY 〈1225〉**

The precision between laboratories.

[NOTE—1. Reproducibility includes contributions from repeatability and all factors that contribute to intermediate precision, as well as any additional contributions from inter-laboratory differences. 2. Reproducibility applies to collaborative studies such as those for standardization or portability of methodology. Depending on the design of the collaborative study, it may be possible to separately describe variance components associated with intra- and inter-laboratory sources of variability.]

**SPECIFICITY 〈1225〉**

The ability to assess unequivocally the analyte in the presence of components that may be expected to be present.
[NOTE—1. Typically these components may include impurities, degradants, matrix, etc. See chapter (1225) for further discussion. 2. This definition is also associated with selectivity in other guidelines for analytical methods. 3. Specificity can mean the measurement of the specific analyte of interest and no other similar analyte.]

**TRUNCATION BIAS**

Bias that occurs when some portion of the distribution of responses is not observed or recorded.

[NOTE—1. When there is truncation bias, the distribution of recorded observations does not match the true distribution of responses. 2. Truncation bias may occur in a bioassay that does not report estimates of log potency outside a set potency range. For example, a sample with a true potency at an edge of this range is expected to fail to yield (report) a potency estimate in approximately half of the assays in which it appears. In this example, the mean of the observed potencies will be biased toward log potency 0.]

**IV. Terms Related to Validation**

**DILUTIONAL LINEARITY (ADAPTED FROM (1225))**

The ability (within a given range) of a bioassay to obtain measured relative potencies that are directly proportional to the true relative potency of the samples.

[NOTE—1. To determine dilutional linearity, sometimes called bioassay analytical linearity, across a range of known relative potency values, analysts examine the relationship between known log potency and mean observed log potency. If that relationship yields an essentially straight line with a y-intercept of 0 and a slope of 1, the assay has direct proportionality. If that plot yields an essentially straight line but either the y-intercept is not 0 or the slope is not 1, the assay has a proportional linear response. 2. To assess whether the slope is (near) 1.0 requires an a priori equivalence or indifference interval. It is not proper statistical practice to test the null hypothesis that the slope is 1.0 against the alternative that it is not 1.0 and then to conclude a slope of 1.0 if this is not rejected. Bioassay analytical linearity is separate from consideration of the shape of the concentration–response curve. Linearity of concentration–response is not a requirement of bioassay analytical linearity since bioassay analytical linearity is possible regardless of the form of the concentration–response curve. 3. Dilutional linearity is further addressed in (1033).]

**QUANTITATION LIMIT (LOWER LIMIT OF QUANTITATION; ADAPTED FROM (1225))**

The lowest known relative potency for which the assay has suitable precision and accuracy.

[NOTE—1. This applies to assay results (log potency) rather than the reportable value. 2. The quantitation limit is not commonly determined for relative potency bioassays. Animal assays with serologic endpoints are examples of the use of this term.]

**RANGE (ADAPTED FROM (1225))**

The interval between the upper and lower known relative potencies (and including those relative potencies) for which the bioassay is demonstrated to have a suitable level of precision, accuracy, and bioassay analytical linearity.

[NOTE—This applies to reportable values (typically a geometric mean) rather than the individual assay results.]

**ROBUSTNESS ((1225))**

A measure of an analytical procedure’s capacity to remain unaffected by small but deliberate variations in method parameters listed in the procedure documentation.

[NOTE—1. Robustness is an indication of a bioassay’s reliability during normal usage. For example, a cell culture assay system that is robust to the passage number of the cells can provide potency values with acceptable accuracy and precision across a consistent range of passage numbers. 2. ICH Q2(R1) states: The evaluation of robustness should be considered during the development phase and depends on the type of procedure under study. It should show the reliability of an analysis with respect to deliberate variations in method parameters. If measurements are susceptible to variations in analytical conditions, the conditions should be suitably controlled or a precautionary statement should be included in the procedure. One consequence of the evaluation of robustness should be that a series of system suitability [q.v.] parameters (e.g., resolution test) is established to ensure that the validity of the analytical procedure is maintained whenever used.]

**VALIDATION, ASSAY**

Assay validation is the process of demonstrating and documenting that the performance characteristics of the procedure and its underlying method meet the requirements for the intended application and that the assay is thereby suitable for its intended use.

[NOTE—Formal validations are conducted prospectively according to a written plan that includes justifiable acceptance criteria on validation parameters. See (1033).]
V. Terms Related to Statistical Design and Analysis

**ANALYSIS OF VARIANCE (ANOVA)**

A statistical tool used to assess contributions to variability from experimental factors.

**BLOCKING**

The grouping of related experimental units in experimental designs.

[NOTE—1. Blocking often is used to reduce the contribution to variability associated with a factor not of primary interest. 2. Blocks may consist, for example, of groups of animals (a cage, a litter, or a shipment), individual 96-well plates, sections of 96-well plates, or whole 96-well plates grouped by analyst, day, or batches of cells. 3. The goal is to isolate, by statistical design and analysis, a systemic effect, such as cage, so that it does not obscure the comparisons of interest.

A complete block design occurs when all levels of a treatment factor (in a bioassay, the primary treatment factors are sample and concentration) can be applied to experimental units for that factor within a single block. Note that the two treatment factors sample and concentration may have different experimental units. For example, if the animals within a cage are all assigned the same concentration but are assigned unique samples, then the experimental unit for concentration is cage and the experimental unit for sample is animal, and cage is a blocking factor for sample. An incomplete block design occurs when the number of levels of a treatment factor exceeds the number of experimental units for that factor within the block.]

**CONFIDENCE INTERVAL**

A random interval produced by a statistical method that contains the true (fixed, but unknown) parameter value with a stated confidence level on repeated application of the statistical method.

[NOTE—See chapter (1010) for more information.]

**CROSSED (AND PARTIALLY CROSSED)**

Two factors are crossed (or fully crossed) if each level of each factor appears with each level of the other factor. Two factors are partially crossed when they are not fully crossed but multiple levels of one factor appear with a common level of the other factor.

[NOTE—1. For example, in a bioassay in which all samples appear at all dilutions, samples and dilutions are (fully) crossed. In a bioassay validation experiment in which two of four analysts each perform assays on the same set of samples on each of six days and a different pair of analysts is used on each day, the analysts are partially crossed with days. 2. Each factor may be applied to different experimental units, and the factors may be both fully crossed and nested (q.v.), creating a split-unit or split-plot design (q.v.). 3. Experiments with factors that are partially crossed require particular care for proper analysis. 4. A randomized complete block design (q.v.) is a design in which the block factor (which often is treated as a random effect) is crossed with the treatment factor (which usually is treated as a fixed effect).]

**DESIGN OF EXPERIMENTS (DOE) [ICH Q8(R2)](5)**

A structured, organized method for determining the relationship between factors that affect a process and the output of that process.

[NOTE—DOE is used in bioassay development and validation; see (1032) and (1033).]

**EQUIVALENCE TEST**

A test to demonstrate equivalence (e.g., similarity or conformance to validation acceptance criteria) of two quantities by conformance to an interval acceptance criterion.

[NOTE—1. An equivalence test differs from most common statistical tests in the nature of the statistical hypotheses. Most common statistical tests are difference tests—that is, the statistical null hypothesis is that of no difference, and the alternative is that there is some difference, without regard to the magnitude or importance of the difference. The difference may be between a characteristic of two populations or between a characteristic of a single population and an accepted value. In equivalence testing the null hypothesis is that the difference is not sufficiently small, and the alternative hypothesis is that the difference is sufficiently small that there is no important difference. In a common statistical difference test one concludes that there is insufficient evidence to establish nonconformance to an acceptance criterion. This may be the result of excess variability and/or an inadequate design. In an equivalence test the conclusion is that the data conform to the acceptance criterion (e.g., slopes are parallel). 2. A common statistical procedure used for equivalence tests is the two one-sided tests (TOST) procedure. 3. The interval acceptance criterion may be one- or two-sided. An example of a one-sided interval is a validation acceptance criterion for a %GCV of not more than XX%]

**EXPECTED MEAN SQUARE**

A mathematical expression of variances estimated by an ANOVA mean square.

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EXPERIMENTAL DESIGN

The structure of assigning treatments to experimental units.

[NOTE—1. Some aspects of experimental design are blocking (q.v.), randomization (q.v.), replication (q.v.), and specific choice of design (cf. (1032)). 2. Important components of experimental design include the number of samples, the number of concentrations, and how samples and concentrations are assigned to experimental units and are grouped into blocks. 3. The experimental design influences which statistical methodology should be used to achieve the analytical objective.]

EXPERIMENTAL UNIT

The smallest unit to which a distinct level of a treatment is randomly allocated.

[NOTE—1. Randomization of treatment factors to experimental units is essential in bioassays. 2. Different treatment factors can be applied to different experimental units. For example, samples may be assigned to rows on a 96-well plate, and dilutions may be assigned to columns on the plate. In this case, rows are the experimental units for samples, columns are the experimental units for concentrations, and wells are the experimental units for the interaction of sample and concentration. 3. An experimental unit must be distinguished from a sampling unit, the smallest unit on which a distinct measurement is recorded (e.g., a well). Because the sampling unit is often smaller than the experimental unit, it is an easy mistake to treat sampling units as if they are experimental units. This mistake is called pseudoreplication (q.v.).]

FACTOR

An assay parameter or operational element that may affect assay response and that varies either within or across assay runs.

[NOTE—In a bioassay there are at least two treatment factors: sample and concentration.

A fixed factor (fixed effect) is a factor that is controllable and deliberately set at specific levels in a bioassay. Inference is made to the levels used in the experiment or intermediate values. Sample and concentration are examples of fixed factors in bioassays.

A random factor (random effect) is one which is generally not controllable and for which its levels represent a sample of ways in which that factor might vary. In a bioassay, the test organisms, plate, and day are often considered random factors. Whether a factor is treated as random or fixed may depend on the experiment and questions asked.]

FACTORIAL DESIGN

An experimental design in which there are multiple factors and the factors are partially or fully crossed.

In a full factorial design, each level of a factor appears with all combinations of levels of all other factors. For example, if factors are reagent batch and incubation time, for a full factorial design all combinations of incubation time and reagent batch must be included.

A fractional factorial design is a reduced design in which each level of a factor appears with only a subset of combinations of levels of all other factors and some factor effects (main effects and/or interactions) are deliberately confounded with other combinations of factor effects. Fractional factorial designs should be carefully considered for screening and optimization purposes. This design can be considered without risk of information loss for validation.

GENERAL LINEAR MODEL

A statistical linear model that relates study factors, which can be continuous or discrete, to experimental responses.

INDEPENDENCE

For two measurements or observations A and B (raw data, assay sets, or relative potencies) to be independent, values for A must be unaffected by B's responses and vice versa.

[NOTE—A consequence of the failure to recognize lack of independence is poor characterization of variance. In practice this means that if two potency or relative potency measurements share a common factor that might influence assay outcome (e.g., analyst, cell preparation, incubator, group of animals, or aliquot of Standard samples), then the correct initial assumption is that these relative potency measurements are not independent. The same concern for lack of independence holds if the two potency or relative potency measurements are estimated together from the same model or are in any way associated without including in the model some term that captures the fact that there are two or more potency measurements. As assay experience is gained, an empirical basis may be established (and monitored) so that it is reasonable to treat potency measurements as independent even if the measurements share a common level of a factor. This is the case when it has been demonstrated that a factor does not have a practically significant effect on long-term bioassay results.]

INTERACTION

Two factors are said to interact if the response to one factor depends on the level of the other factor.

LEVEL

A location on the scale of measurement of a factor.

[NOTE—1. Factors have two or more distinct levels. For example, if a bioassay validation experiment employs three values of incubation time and two batches of a key reagent, the levels are the three times for the factor incubation time and the two
batches for the factor batch. 2. Levels of a factor in a bioassay may be quantitative, such as concentration, or categorical, such as sample (i.e., test and reference).

LOGNORMAL DISTRIBUTION
A distribution of values (assay responses or potencies) where the logarithms of the values have a normal distribution. [NOTE—1. Most relative potency bioassay measurements are lognormally distributed. 2. The lognormal is a skewed distribution characterized by increased variability with increased level of response.]

MEAN SQUARE
A calculation in ANOVA representing the variability associated with an experimental factor.

MIXED-EFFECTS MODEL
A statistical model that includes both fixed and random effects.

MODELING, STATISTICAL
The mathematical specification of the relationship between inputs (Xs) and outputs (Ys) of a process, e.g., the concentration–response relationship in bioassay or the modeling of the effects of important sources of variation on potency measurement. [NOTE—1. Modeling includes methods to capture the dependence of the response on the samples, concentration, experimental units, and groups or blocking factors in the assay configuration. 2. Modeling of bioassay data includes making many choices, some of which are driven by the assay design and data. For continuous data there is a choice between linear and nonlinear models. For discrete data there is a choice among logit/log models within a larger family of generalized linear models. In limiting dilution assays, published literature advocates Poisson models and Markov chain binomial models. One can use either fixed-effects models or mixed-effects models for bioassay data. On the one hand, the fixed-effects models are more widely available in software and are somewhat less demanding for statisticians to set up. On the other hand, mixed models have advantages over fixed ones: they are more accommodating of missing data and, more importantly, can allow each block to have different slopes, asymptotes, median effective concentrations required to induce a 50% effect (EC50), or relative potencies. Particularly when the analyst is using straight-line models fitted to nonlinear responses or assay systems in which the concentration–response curve varies from block to block, the mixed model captures the behavior of the assay system in a much more realistic and interpretable way. 3. It is essential that any modeling approach for bioassay data should use all available data simultaneously to estimate the variation (or, in a mixed model, each of several sources of variation). It may be necessary to transform the observations before this modeling to include a variance model or to fit a means model (in which there is a predicted effect for each combination of sample and concentration) to get pooled estimate(s) of variation.]

NESTED
A factor A is nested within another factor B if the levels of A are different for every level of B. [NOTE—1. For example, in a bioassay validation experiment two analysts may perform assays on five days each. If the calendar days for the first analyst are distinct from those of the second analyst, days are nested within analyst. 2. Nested factors have a hierarchical relationship. 3. For two factors to be nested they must satisfy the following: (a) they are applied to different-sized experimental units; (b) the larger experimental unit contains more than one of the smaller experimental units; and (c) the factor applied to the smaller experimental unit is not fully crossed (q.v.) with the factor applied to the larger experimental unit. When conditions (a) and (b) are satisfied and the factors are partially crossed, then the experiment is partially crossed and partially nested. Experiments with this structure require particular care for proper analysis.]

PARALLELISM (OF CONCENTRATION–RESPONSE CURVES)
A quality in which the concentration–response curves of the Test sample and the Reference Standard are identical in shape and differ only by a horizontal difference that is a constant function of relative potency. [NOTE—1. When Test and Reference preparations are similar (q.v.) and assay responses are plotted against log concentrations, the resulting curve for the Test preparation will be the same as that for the Standard but will be shifted horizontally by an amount that is the logratio of the relative potency. Because of this relationship, similarity (q.v.) is often equated with parallelism but they are not the same. See section 3.5, Slope-Ratio Concentration–Response Models, in chapter (1034), in which similar samples have concentration–response relationships with a common (or nearly common) y-intercept but may differ in their slopes. 2. In practice, it is not possible to demonstrate that the shapes of two curves are identical. Instead, the two curves are shown to be sufficiently algebraically similar (equivalent) in shape. Note that similar should be interpreted as “we have evidence that the two curves are close enough in shape” rather than “we do not have evidence that the two curves differ in shape.” 3. The assessment of parallelism depends on the type of function used to fit the response curve. Parallelism for a nonlinear assay using a four-parameter logistic fit means that (a) the slopes of the rapidly changing parts of the Test and Reference Standard curves (that is, slope at a tangent to the curve where the first derivative is at a maximum) should be similar, and (b) the upper and lower asymptotes of the response curves (plateaus) should be similar. For straight-line analysis, the slopes of the lines should be similar.]

POINT ESTIMATE
A single-value estimate obtained from statistical calculations.
PSEUDOREPLICATION

The misidentification of samples from experimental units as independent and thus true replicates when they actually are not independent.

[NOTE—1. Pseudoreplication results in incorrect inferences because of the incorrect assignment of variability and the appearance of more replicates than are actually present. 2. Lack of recognition of pseudoreplication is critical because it is an easy mistake to make, and the consequences can be serious. For example, pseudoreplicates commonly arise when analysts make a dilution series for each sample in tubes (the dilution series can be made with serial dilutions, single-point dilutions, or any convenient dilution scheme). The analyst then transfers each dilution of each sample to several wells on one or more assay plates. The wells are then pseudoreplicates because they are simply aliquots of a single dilution process and thus are not representative of independent preparations. 3. A simple way to analyze data from pseudoreplicates is to average over the pseudoreplicates (if a transformation of the observed data is used, the transformation should be applied before averaging over pseudoreplicates) before fitting any concentration–response model. In many assay systems, averaging over pseudoreplicates leaves the assay without any replication. A more complex way to use data containing pseudoreplicates is to use a mixed model that treats the pseudoreplicates as a separate random effect. Although pseudoreplication normally is of little value, it can be advantageous when two conditions are satisfied: (a) the pseudoreplicate (e.g., well-to-well) variation is very large compared to the variation associated with replicates; and (b) the cost of pseudoreplicates is much lower than the cost of replicate experimental units.]

P VALUE (SIGNIFICANCE PROBABILITY)

The probability of observing, in repeated trials, that an experimental outcome is as different or more different than that observed if the null hypothesis is true.

[NOTE—1. More different means further from the null hypothesis. 2. Commonly, $P<0.05$ is taken as a threshold for indicating statistically significant differences, although any value for the threshold may be used. Bases for choosing the threshold are the risks (costs) of making a wrong decision; see type I error and type II error.]

RANDOMIZATION

A process of assignment of treatment to experimental units based on chance so that all equal-sized subgroups of units have an equal chance of receiving a given treatment.

[NOTE—1. The chance mechanism may be an unbiased physical process (rolling unbiased dice, flipping coins, drawing from a well-mixed urn), random-number tables, or computer-generated randomized numbers. Care must be taken in the choice and use of method. Good practice is to use a validated computerized random-number generator. 2. The use of randomization can help to prevent systematic error from becoming associated with particular samples or a dilution pattern and causing bias. For example, in 96-well bioassays, plate effects can be substantial and can cause bias in observed responses or summary measures. In animal studies, a variety of factors associated with individual animals can influence responses. If extraneous factors that influence either plate assays or animal assays are not routinely demonstrated to have been eliminated or minimized so as to be negligible, randomization is essential to obtaining unbiased data required for the calculation of true potency. 3. Randomization is a good practice even when there is evidence that operational factors (e.g., location, time, reagent lot) have little or no effect on the assay system. While randomization may not protect an individual assay (or perhaps a block of an assay) from a (perhaps newly) important operational factor, randomization provides assurance that results from a collection of assays are not biased due to operational factors.]

REPLICATION

A process in which multiple independent experimental units receive the same level of a treatment factor.

[NOTE—1. The purpose of replication is to minimize the effects of uncontrollable sources of random variability. 2. Replication can occur either completely at random or across blocks. Generally, replication within blocks is pseudoreplication (q.v.). 3. Replication of factors that contribute most greatly to variability, or factors that are at the highest levels in a nested layout, usually result in the most effective reduction of random variability.]

TRUE REPLICATES

Samples based on independent experimental units.

STANDARD ERROR OF ESTIMATE

A measure of uncertainty of an estimate of a reportable value or other parameter estimate because of sampling variation.

[NOTE—1. In bioassay the focus is on the precision (standard error) of the relative potency. 2. Standard errors can be made smaller with additional replication. 3. Technically, the standard error of an estimate is the standard deviation of the sampling distribution of the estimate. The term standard error is used to distinguish between this usage of standard deviation (that depends on sample size) and the common laboratory usage in which standard deviation (or coefficient of variation) is used to characterize the precision of individual measurements obtained from a procedure. This latter precision does not depend on sample size.]
STATISTICAL PROCESS CONTROL

A set of statistical methods used to monitor shifts and trends in a process.

TYPE I ERROR

The error in statistical hypothesis testing that the alternative hypothesis is accepted when it is false.

[NOTE—The probability of a type I error usually is denoted by \( \alpha \).]

TYPE II ERROR

The error in statistical hypothesis testing that the alternative hypothesis is rejected when it is true.

[NOTE—The probability of a type II error usually is denoted by \( \beta \).]

VARIANCE COMPONENT ANALYSIS

A statistical analysis that partitions contributions made to total variability by components associated with influential assay factors, e.g., analyst, day, or instrument.

(1032) DESIGN AND DEVELOPMENT OF BIOLOGICAL ASSAYS

1. INTRODUCTION

1.1 Purpose and Scope

General chapter Design and Development of Biological Assays (1032) presents methodology for the development of bioassay procedures that have sound experimental design, that provide data that can be analyzed using well-founded statistical principles, and that are fit for their specific use.

General chapter (1032) is one of a group of five general chapters that focus on relative potency assays, in which the activity of a Test material is quantified by comparison to the activity of a Standard material. However, many of the principles can be applied to other assay systems.

This general chapter is intended to guide the design and development of a bioassay for a drug substance or product intended for commercial distribution. Although adoption of this chapter’s recommended methods may be resource intensive during assay development, early implementation can yield benefits. Lastly, the perspectives and methods described herein are those recommended from among the many alternatives which contemporary bioassay theory and practice offers.

FOCUS ON RELATIVE POTENCY

Because of the inherent variability in biological test systems (including that from animals, cells, instruments, reagents, and day-to-day and between-lab), an absolute measure of potency is more variable than a measure of activity relative to a Standard. This has led to the adoption of the relative potency methodology. Assuming that the Standard and Test materials are biologically similar, statistical similarity (a consequence of the Test and Standard similarity) should be present, and the Test sample can be expected to behave like a concentration or dilution of the Standard. Relative potency is a unitless measure obtained from a comparison of the dose-response relationships of Test and Standard drug preparations. For the purpose of the relative comparison of Test to Standard, the potency of the Standard is usually assigned a value of 1 (or 100%). The Standard can be a material established as such by a national (e.g., USP) or international (e.g., WHO) organization, or it could be an internal Standard.

1.2 Audience

This chapter is intended for both the practicing bioassay analyst and the statistician who are engaged in developing a bioassay. The former will find guidance for implementing bioassay structure and methodology to achieve analytical goals while reliably demonstrating the biological activity of interest, and the latter will gain insights regarding the constraints of biology that can prove challenging to balance with a rigorous practice of statistics.

2. BIOASSAY FITNESS FOR USE

To evaluate whether an assay is fit for use, analysts must specify clearly the purpose(s) for performing the bioassay. Common uses for a bioassay include lot release of drug substance (active pharmaceutical ingredient) and drug product; assessment of stability; qualification of Standard and other critical reagents; characterization of process intermediates and formulations; characterization of contaminants and degradation products; and support of changes in the product production process. The relative accuracy, specificity, precision, and robustness requirements may be different for each of these potential uses. It is a good strategy to develop and validate a bioassay to support multiple intended uses; for example, a bioassay primarily developed for batch release may serve other purposes. Decisions about fitness for use are based on scientific and statistical considerations, as well as practical considerations such as cost, turnaround time, and throughput requirements for the assay.

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When assays are used for lot release, a linear-model bioassay may allow sufficient assessment of similarity. For bioassays used to support stability, comparability, to qualify reference materials or critical reagents, or in association with changes in the production or assay processes, it is generally useful to assess similarity using the entire concentration–response curve, including the asymptotes (if present).

### 2.1 Process Development

Bioassays are generally required in the development and optimization of product manufacturing, including formulation and scale-up processes. Bioassays can be used to evaluate purification strategies, optimize product yield, and measure product stability. Because samples taken throughout the process are often analyzed and compared, sample matrix effects that may affect assay response should be carefully studied to determine an assay’s fitness for use. For relative potency measures, the Standard material may require dilution into a suitable matrix for quantitation. The bioassay’s precision and accuracy should be sufficient for measuring process performance or for assessing and comparing the stability of candidate formulations.

### 2.2 Process Characterization

Bioassays may be performed to assess the effect on drug potency associated with different stages of drug manufacture or with changes in the manufacturing process (e.g., to demonstrate product equivalence before and after process changes are made). Bioassays used in this type of application may be qualitative or quantitative.

### 2.3 Product Release

Bioassays are used to evaluate the potency of the drug before commercial product release. To the extent possible, the assay should reflect or mimic the product’s known or intended mechanism of action. If the bioassay does not include the functional biology directly associated with the mechanism of action, it may be necessary to demonstrate a relationship between the bioassay’s estimated potency determinations and those of some other assay that better or otherwise reflects putative functional activity.

For product-release testing, product specifications are established to define a minimum or range of potency values that are acceptable for product. The precision of the reportable value from the bioassay must support the number of significant digits listed in the specification (see general chapter Biological Assay Validation (1033)), and, in conjunction with relative accuracy, support the specification range. In order to meet these specifications, manufacturing quality control will have sufficiently narrow product release specifications in order to accommodate any loss of activity due to instability and uncertainty in the release assay.

### 2.4 Process Intermediates

Bioassay assessment of process intermediates can provide information regarding specificity. Formulation and fill strategies may rely on bioassays in order to ensure that drug product, including that in final container, will meet its established specifications. For example, unformulated bulk materials may be held and evaluated for potency. Bulks may be pooled with other bulk lots, diluted, or reworked based on the potency results. For these types of applications, the bioassay must be capable of measuring product activity in different matrices. In some cases, a separate Standard material is made and is used to calculate relative potency for the process intermediate.

### 2.5 Stability

The potency assay may be used to assess biotechnology and vaccine product stability. Information from stability studies, performed during development under actual and/or accelerated or stressed storage conditions, may be used to establish shelf life duration as well as to identify and estimate degradation products and degradation rates. Post licensure stability studies may be used to monitor product stability. Knowledge of both short-term and long-term variability of the bioassay is important to assure an acceptable level of uncertainty in potency measures obtained.

### 2.6 Qualification of Reagents

The quantitative characterization of a new Standard requires an accurate and precise measurement of the new Standard’s biological activity. This measurement is used either to establish that the new Standard lot is equivalent to the previous lot or to assign it a label potency to which Test samples can be compared. Additional replication (beyond routine testing) may be required to achieve greater precision in the potency measurement of the new Standard material. Additionally, the bioassay may be used to qualify a cell culture reagent such as fetal bovine serum. The fitness for use in such cases is tied to the ability of the assay to screen reagent lots and to ensure that lots that may bias or compromise the relative potency measurements are not accepted.

### 2.7 Product Integrity

Biotechnology, biological, and vaccine products may contain a population of heterogeneous material, including the intended predominant product material. Some process impurities and degradation products may be active, partially active, inactive in, or antagonistic to, the response measured in the bioassay. For product variants or derivatives for which changes in structure or relative composition may be associated with subtle yet characteristic changes in the bioassay response (e.g., change in slope or asymptote), the bioassay may be useful in the detection and measurement of these variants or derivatives. Studies that identify
characteristic changes associated with variants of the intended product help ensure consistent product performance. Whenever practical, the bioassay should be accompanied by orthogonal methods that are sensitive to product variants, process impurities, and/or degradation products.

3. BIOASSAY FUNDAMENTALS

3.1 In Vivo Bioassays

In vivo potency assays are bioassays in which sets of dilutions of the Standard and Test materials are administered to animals and the concentration–response relationships are used to estimate potency. For some animal assays, the endpoint is simple (e.g., rat body weight gain assay for human growth hormone or rat ovarian weight assay for follicle stimulating hormone), but others require further processing of samples collected from treated animals (e.g., reticulocyte count for erythropoietin, steroidogenesis for gonadotropins, neutrophil count for granulocyte colony stimulating factor, or antibody titer after administration of vaccines). With the advent of cell lines specific for the putative physiological mechanism of action (MOA), the use of animals for the measurement of potency has substantially diminished. Cost, low throughput, ethical, and other practical issues argue against the use of animal bioassays. Regulatory agencies have encouraged the responsible limitation of animal use whenever possible (see The Interagency Coordinating Committee on the Validation of Alternative Methods, Mission, Vision, and Strategic Priorities; February 2004). When in vitro activity is not strongly associated with in vivo activity (e.g., EPO), the combination of an in vitro cell-based assay and a suitable physicochemical method (e.g., IEF, glycan analysis) may substitute for in vivo assays. However, a need for in vivo assays may remain when in vitro assays cannot detect differences that are critical in regard to a drug’s intended biological function.

Animals’ physiological responses to biological drugs (including vaccines) may predict patients’ responses. Selection of animal test subjects by species, strain, gender, and maturity or weight range is guided by the goal of developing a representative and sensitive model with which to assess the activity of Test samples.

Some assay methods lend themselves to the use of colony versus naive animals. For example, pyrogen and insulin testing benefit from using experienced colony rabbits that provide a reliable response capacity. If animals recently introduced to the colony fail to respond as expected after several administrations of a compound, they should be culled from the colony so they do not cause future invalid or indeterminate assay results. In the case of assaying highly antigenic compounds for pyrogens, however, naive animals should be used to avoid generating inaccurate or confounded results. Other colony advantages include common controlled environmental conditions (macro/room, and micro/rack), consistent feeding schedule, provision of water, and husbandry routine.

Historical data including colony records and assay data can be used to identify factors that influence assay performance. The influence of biasing factors can be reduced by applying randomization principles such as distribution of weight ranges across dose groups, group assignments from shipping containers to different cages, or use of computer-generated or deck patterns for injection/dosing. A test animal must be healthy and have time to stabilize in its environment to be suitable for use in a bioassay. Factors that combine to influence an animal’s state of health include proper nutrition, hydration, freedom from physical and psychological stressors, adequate housing sanitation, controlled light cycle (diurnal/nocturnal), experienced handling, skillful injections and bleedings, and absence of noise or vibration. Daily observation of test animals is essential for maintenance of health, and veterinary care must be available to evaluate issues that have the potential to compromise the validity of bioassay results.

3.2 Ex Vivo Bioassays

Cells or tissues from human or animal donors can be cultured in the laboratory and used to assess the activity of a Test sample. In the case of cytokines, the majority of assays use cells from the hematopoietic system or subsets of hematopoietic cells from peripheral blood such as peripheral blood mononuclear cells or peripheral blood lymphocytes. For proteins that act on solid tissues, such as growth factors and hormones, specific tissue on which they act can be removed from animals, dissociated, and cultured for a limited period either as adherent or semi-adherent cells. Although an ex vivo assay system has the advantage of similarity to the natural milieu, it may also suffer from substantial donor-to-donor variability, as well as challenging availability of appropriate cells.

Bioassays that involve live tissues or cells from an animal (e.g., rat hepatocyte glucagon method) require process management similar to that of in vivo assays to minimize assay variability and bias. The level of effort to manage bias (e.g., via randomization) should be appropriate for the purpose of the assay. Additional factors that may affect assay results include time of day, weight or maturity of animal, anesthetic used, buffer components/reagents, incubation bath temperature and position, and cell viability.

3.3 In Vitro (Cell-Based) Bioassays

Bioassays using cell lines that respond to specific ligands or infectious agents can be used for lot-release assays. These cell lines can be derived from tumors, immortalized as factor-dependent cell lines, or engineered cell lines transfected with appropriate receptors. Additionally, nontransformed cell lines which can be maintained over a sufficient number of passages (e.g., fibroblasts) may also be used. Regardless of cell line, there is an expectation of adequately equivalent potency response through some number of continuous passages. Advances in recombinant DNA technology and the understanding of cellular signaling mechanisms have allowed the generation of engineered cell lines with improved response, stable expression of receptors and signaling mechanisms, and longer stability. The cellular responses to the protein of interest depend on the drug’s MOA and the duration of exposure. Such responses include cell proliferation, cell killing, antiviral activity, differentiation, cytokine/mediator secretion, and enzyme activation. Assays involving these responses may require incubation of the cells over
several days, during which time contamination, uneven evaporation, or other location effects may arise. Comparatively rapid responses based on an intracellular signaling mechanism—such as second messengers, protein kinase activation, or reporter gene expression—have proven acceptable to regulatory authorities. Lastly, most cell lines used for bioassays express receptors for multiple cytokines and growth factors. This lack of specificity may not be detrimental if the Test sample’s specificity is demonstrated.

Cell-based bioassay design should reflect knowledge of the factors that influence the response of the cells to the active analyte. Response variability is often reflected in parameters such as slope, EC₅₀ of the concentration–response curve, or the response range (minimum minus minimum response). Even though relative potency methodology minimizes the effects on potency estimates of variation in these parameters among assays, and among blocks within an assay, such response variability can make an assay difficult to manage (i.e., it may be difficult to assess system suitability). Hence, while assay development should be focused primarily on the properties of potency, efforts to identify and control variation in the concentration–response relationship are also appropriate. For blocked assays (e.g., multiple cell culture plates in an assay) with appreciable variation in curve shape among blocks, an analysis that does not properly include blocks will yield inflated estimates of within-assay variation, making similarity assessment particularly difficult. Two strategies are available for addressing variation among blocks: one, a laboratory effort to identify and control sources of variation and two, a statistical effort to build and use a blocked design and analysis. Combining these strategies can be particularly effective.

The development of a cell-based bioassay begins with the selection or generation of a cell line. An important first step when developing a cell-based assay to assess a commercial product is to verify that the cell line of interest is not restricted to research use only. To ensure an adequate and consistent supply of cells for product testing, a cell bank should be generated if possible. To the extent possible, information regarding functional and genetic characteristics of the bioassay’s cell line should be documented, including details of the cell line’s history from origin to banking. For example, for a recombinant cell line this might include the identification of the source of the parental cell line (internal cell bank, external repository, etc.), of the DNA sequences used for transfection, and of the subsequent selection and functional testing regimen that resulted in selection of the cell line. Ideally, though not always practical, sufficient information is available to permit recreation of a similar cell line if necessary. Pertinent information may include identity (e.g., isoenzyme, phenotypic markers, genetic analysis); morphology (e.g., archived photographic images); purity (e.g., mycoplasma, bacteria, fungus and virus testing); cryopreservation; thaw and culture conditions (e.g., media components, thaw temperature and method, methods of propagation, seeding densities, harvest conditions); thaw viability (immediately after being frozen and after time in storage); growth characteristics (e.g., cell doubling times); and functional stability (e.g., ploidy).

Cell characterization and vigilance regarding aspects of assay performance that reflect on cell status are necessary to ensure the quality and longevity of cell banks for use in the QC environment. The general health and metabolic state of the cells at the time of bioassay can substantially influence the test results. After a cell line has been characterized and is ready for banking, analysts typically prepare a two-tiered bank (Master and Working). A Master Cell Bank is created as the source for the Working Cell Bank. The Working Cell Bank is derived by expansion of one or more vials of the Master Cell Bank. The size of the banks depends on the growth characteristics of the cells, the number of cells required for each assay, and how often the assay will be performed. Some cells may be sensitive to cryopreservation, thawing, and culture conditions, and the banks must be carefully prepared and characterized before being used for validation studies and for regular use in the QC laboratory.

There follow factors that may affect bioassay response and the assessment of potency, that are common to many cell-based bioassays: cell type (adherent or nonadherent); cell thawing; plating density (at thaw and during seed train maintenance) and confluence (adherent cells); culture vessels; growth, staging, and assay media; serum requirements (source, heat inactivation, gamma irradiation); incubation conditions (temperature, CO₂, humidity, culture times from thaw); cell harvesting reagents and techniques (for adherent cells, method of dissociation); cell sorting; cell counting; determination of cell health (growth rate, viability, yield); cell passage number and passing schedule; cell line stability (genetic, receptor, marker, gene expression level); and starvation or stimulation steps. This list is not exhaustive, and analysts with comprehensive understanding and experience with the cell line should be involved during assay development. These experienced individuals should identify factors that might influence assay outcomes and establish strategies for an appropriate level of control whenever possible.

3.4 Standard

The Standard is a critical reagent in bioassays because of the necessity to have a reliable material to which a Test preparation can be quantitatively compared. The Standard may be assigned a unitage or specific activity that represents fully (100%) potent material. Where possible, a Standard should be representative of the samples to be tested in the bioassay. Testing performed to qualify a Standard may be more rigorous than the routine testing used for lot release.

A Standard must be stored under conditions that preserve its full potency for the intended duration of its use. To this end, the Standard may be stored under conditions that are different from the normal storage of the drug substance or drug product. These could include a different temperature (e.g., −70° or −20°, instead of 2°–8°), a different container (e.g., plastic vials instead of syringes), a different formulation (e.g., lyophilized formulation or the addition of carrier proteins such as human serum albumin, stabilizers, etc.). The Standard material should be tested for stability at appropriate intervals. System suitability criteria of the bioassay such as maximum or background response, EC₅₀ slope, or potency of assay control may be used to detect change in the activity of the Standard. Accelerated stability studies can be performed to estimate degradation rates and establish recognizable characteristics of Standard instability. At later stages in clinical development, the Standard may be prepared using the manufacturing process employed in pivotal clinical trials. If the Standard formulation is different from that used in the drug product process, it is important to demonstrate that the assay’s assessment of similarity and estimate of potency is not sensitive to the differences in formulation. An initial Standard may be referred to as the Primary Standard. Subsequent Standards can be prepared using current manufacturing processes and can be designated Working Standards. Separate SOPs may be required for establishing these standards for each product. Bias in potency measurements sometimes can arise if the activity of the Standard gradually changes. Also, loss of similarity may be observed if, with time, the Standard undergoes changes in glycosylation. It is prudent to archive aliquots of each Standard lot for assessment of comparability with later Standards and for the investigation of assay drift.
4. STATISTICAL ASPECTS OF BIOASSAY FUNDAMENTALS

The statistical elements of bioassay development include the type of data, the measure of response at varying concentration, the assay design, the statistical model, pre-analysis treatment of the data, methods of data analysis, suitability testing, and outlier analysis. These form the constituents of the bioassay system that will be used to estimate the potency of a Test sample.

4.1 Data

Fundamentally, there are two bioassay data types: quantitative and quantal (categorical). Quantitative data can be either continuous (not limited to discrete observations; e.g., collected from an instrument), count (e.g., plaque-forming units), or discrete (e.g., endpoint dilution titers). Quantal data are often dichotomous; for example, life/death in an animal response model or positivity/negativity in a plate-based infectivity assay that results in destruction of a cell monolayer following administration of an infectious agent. Quantitative data can be transformed to quantal data by selecting a threshold that distinguishes a positive response from a negative response. Such a threshold can be calculated from data acquired from a negative control, as by adding (or subtracting) a measure of uncertainty (such as two or three times the standard deviation of negative control responses) to the negative control average. Analysts should be cautious about transforming quantitative data to quantal data because this results in a loss of information.

4.2 Assumptions

A key assumption for the analysis of most bioassays is that the Standard and Test samples contain the same effective analyte or population of analytes and thus may be expected to behave similarly in the bioassay. This is termed similarity. As will be shown in more detail in the general chapter Analysis of Biological Assays (1034) for specific statistical models, biological similarity implies that statistical similarity is present (for parallel-line and parallel-curve models, the Standard and Test curves are parallel; for slope-ratio models, the Standard and Test lines have a common intercept). The reverse is not true. Statistical similarity (parallel lines, parallel curves, or common intercept, as appropriate) does not ensure biological similarity. However, failure to satisfy statistical similarity may be taken as evidence against biological similarity. The existence of a Standard–Test sample pair that passes the assessment of statistical similarity is thus a necessary but not sufficient condition for the satisfaction of the key assumption of biological similarity. Biological similarity thus remains, unavoidably, an assumption. Departures from statistical similarity that are consistent in value across replicate assays may be indicative of matrix effects or of real differences between Test and Standard materials. This is true even if the departure from statistical similarity is sufficiently small to support determination of a relative potency.

In many assays multiple compounds will yield similar concentration–response curves. It may be reasonable to use a biological assay system to describe or even compare response curves from different compounds. But it is not appropriate to report relative potency unless the Standard and Test samples contain only the same active analyte or population of analytes. Biological products typically exhibit lot-to-lot variation in the distribution of analytes (i.e., most biological products contain an intended product and, at acceptable low levels, some process contaminants that may be active in the bioassay). Assessment of similarity is then, at least partially, an assessment of whether the distribution of analytes in the Test sample is close enough to that of the distribution in the Standard sample for relative potency to be meaningful; that is, the assay is a comparison of like to like. When there is evidence (from methods other than the bioassay) that the Standard and Test samples do not contain the same active compound(s), the assumption of biological similarity is not satisfied, and it is not appropriate to report relative potency.

Other common statistical assumptions in the analysis of quantitative bioassays are constant variance of the responses around the fitted model (see section 4.3 Variance Heterogeneity, Weighting, and Transformation for further discussion), normally distributed residuals (a residual is the difference between an observed response and the response predicted by the model), and independence of the residuals.

Constant variance, normality, and independence are interrelated in the practice of bioassay. For bioassays with a quantitative response, a well-chosen data transformation may be used to obtain approximately constant variance and a nearly normal distribution of residuals. Once such transformation has been imposed, the remaining assumption of independence then remains to be addressed via reflection of the assay design structure in the analysis model. Independence of residuals is important for assessing system and sample suitability.

4.3 Variance Heterogeneity, Weighting, and Transformation

Simple analysis of quantitative bioassay data requires that the data be approximately normally distributed with near constant variance across the range of the data. For linear and nonlinear regression models, the variance referred to here is the residual variance from the fit of the model. Constant variance is often not observed; variance heterogeneity may manifest as an increase in variability with increase in response. If the variances are not equal but the data are analyzed as though they are, the estimate of relative potency may still be reasonable; however, failure to address nonconstant variance around the fitted concentration–response model results in an unreliable estimate of within-assay variance. Further, the assessment of statistical similarity may not be accurate, and standard errors and confidence intervals for all parameters (including a Fieller’s Theorem-based interval for the relative potency) should not be used. Confidence intervals for relative potency that combine potency estimates from multiple assays may be erroneous if within-assay error is used for confidence interval calculation.

Constancy of variance may be assessed by means of residual plots, Box-Cox (or power law) analysis, or Levene’s test. With Levene’s test, rather than relying on the p value, change in the statistic obtained is useful as a basis for judging whether homogeneity is improved or worsened. Variance is best assessed on a large body of assay data. Using only the variance among replicates from the current assay is not appropriate, because there are too few data to properly determine truly representative variances specific to each concentration. Data on variance is sparse during development; it is prudent to re-assess variance during validation and to monitor it periodically during ongoing use of the assay.
Two methods used to mitigate variance heterogeneity are transformation and weighting. Lack of constant variance can be addressed with a suitable transformation. Additionally, transformation can improve the normality of residuals and the fit of some statistical models to the data. A transformation should be chosen from an assay system during development, checked during validation, used consistently in routine assay practice, and checked periodically. Bioassay data are commonly displayed with log-transformed concentration; slope-ratio assays are displayed with concentration on the original scale.

Transformation may be performed to the response data as well as to the concentration data. Common choices for a transformation of the response include log, square root (for counts), reciprocal, and, for count data with known asymptotes, log of the percent of maximum response. Log transformations are commonly used, as they may make nearly linear a useful segment of the concentration–response relationship, and because of the ease of transforming back to the original scale for interpretation. A log–log fit may be performed on data exhibiting nonlinear behavior. Other alternatives are available; i.e., data may be transformed by the inverse of the Power of the Mean (POM) function. A POM coefficient of \( k = 2 \) corresponds to a log transformation of the data. For further discussion of relationships between log-transformed and untransformed data, see Appendix in the general chapter Biological Assay Validation (1033).

Note that transformation of the data requires re-evaluation of the model used to fit the data. From a statistical perspective there is nothing special about the original scale of measurement; any transformation that improves accordance with assumptions is acceptable. Analysts should recognize, however, that transformations, choice of statistical model, and choice of weighting scheme are interrelated. If a transformation is used, that may affect the choice of model. That is, transforming the response by a log or square root, for example, may change the shape of the response curve, and, for a linear model, may change the range of concentrations for which the responses are nearly straight and nearly parallel.

For assays with non-constant variance, a weighted analysis may be a reasonable option. Though weighting cannot address lack of residual normality, it is a valid statistical approach to placing emphasis on more precise data. Ideally, weights may be based on the inverse of the predicted within-assay (or within-block) variance of each response where the predictors of variance are independent of observations observed in a specific assay.

In practice, many bioassays have relatively large variation in log EC\(_{50}\) (compared to the variation in log relative potency) among assays (and sometimes among blocks within assay). If not addressed in the variance model, this variation in log EC\(_{50}\) induces what appears to be large variation in response near the mean log EC\(_{50}\), often yielding too-low weights for observations near the EC\(_{50}\).

If the assay is fairly stable (low variability in EC\(_{50}\)), an alternative is to look at variance as a function of concentration. While not ideal, an approach using concentration-dependent variances may be reasonable when the weights are estimated from a large number of assays, the variances are small, any imbalance in the number of observations across concentrations is addressed in the variance model, and there are no unusual observations (outliers). This possibility can be examined by plotting the response variance at each concentration (preferably pooled across multiple assays) against concentration and then against a function of concentration (e.g., concentration squared). Variance will be proportional to the function of concentration where this plot approximates a straight line. The apparent slope of this line is informative, in that a horizontal line indicates no weighting is needed. If a function that yields a linear plot can be found, then the weights are taken as proportional to the reciprocal of that function. There may be no such function, particularly if the variation is higher (or lower) at both extremes of the concentration range studied.

Whether a model or historical data are used, the goal is to capture the relative variability at each concentration. It is not necessary to assume that the absolute level of variability of the current assay is identical to that of the data used to determine the weighting, but only that the ratios of variances among concentrations are consistent with the historical data or the data used to determine the variance function.

Appropriate training and experience in statistical methods are essential in determining an appropriate variance-modeling strategy. Sources of variability may be misidentified if the wrong variance model is used. For example, data may have constant variation throughout a four-parameter logistic concentration–response curve but can also have appreciable variation in the EC\(_{50}\) parameter from block to block within the assay, or from assay to assay. If the between-block or between-assay variability is not recognized, this assay can appear to have large variation in the response for concentrations near the long-term average value of the EC\(_{50}\). A weighted model with low weights for concentrations near the EC\(_{50}\) would misrepresent a major feature of such an assay system.

### 4.4 Normality

Many statistical methods for the analysis of quantitative responses assume normality of the residuals. If the normality assumption is not met, the estimate of relative potency and its standard error may be reasonable, but suitability tests and a confidence interval for the relative potency estimate may be invalid. Most methods used in this chapter are reasonably robust to departures from normality, so the goal is to detect substantial nonnormality. During assay development, in order to discover substantial departure from normality, graphical tools such as a normal probability plot or a histogram (or something similar like stem-and-leaf or box plots) of the residuals from the model fit may be used. The histogram should appear unimodal and symmetric. The normal probability plot should approximate a straight line; a normal probability plot that is not straight (e.g., curved at one end, both ends, or in the middle) indicates the presence of nonnormality. A pattern about a straight line is an indication of nonnormality. Nonnormal behavior may be due to measurements that are log normal and show greater variability at higher levels of response. This may be seen as a concave pattern in the residuals in a normal plot.

Statistical tests of normality may not be useful. As per the previous discussion of statistical testing of constancy of variance, change of the value of a normality test statistic, rather than reliance on a p value, is useful for judging whether normality is improved or worsened. As for variance assessment, evaluate normality on as large a body of assay data as possible during development, re-assigns during validation, and monitor periodically during ongoing use of the assay. Important departures from normality can often be mitigated with a suitable transformation. Failure to assess and mitigate important departure from normality carries the risks of disabling appropriate outlier detection and losing capacity to obtain reliable estimates of variation.
4.5 Linearity of Concentration–Response Data

Some bioassay analyses assume that the shape of the concentration–response curve is a straight line or approximates a straight line over a limited range of concentrations. In those cases, a linear-response model may be assessed to determine if it is justified for the data in hand. Difference testing methods for assessing linearity face the same problems as do difference testing methods applied to parallelism—more data and better precision make it more likely to detect nonlinearity. Because instances in which lack of linearity does not affect the potency estimate are rare, analysts should routinely assess departure from linearity if they wish to use a linear-response model to estimate potency.

If an examination of a data plot clearly reveals departure from linearity, this is sufficient to support a conclusion that linearity is not present. High data variability, however, may mask departure from linearity. Thus a general approach for linearity can conform to that for similarity, developed more elaborately in section 4.7 Suitability Testing, Implementing Equivalence Testing for Similarity (parallelism).

1. Specify a measure of departure from linearity which can either combine across samples or be sample specific. Possibilities include the nonlinearity sum of squares or quadratic coefficients.
2. Use one of the four approaches in Step 2 of Implementing Equivalence Testing for Similarity (parallelism) to determine, during development, a range of acceptable values (acceptance interval) for the measure of nonlinearity.
3. Determine a 90% two-sided confidence interval on the measure on nonlinearity, following the Two One-Sided Test (TOST) procedure, and compare the result to the acceptance interval as determined in (2).

Often a subset of the concentrations measured in the assay will be selected in order to establish a linear concentration–response curve. The subset may be identified graphically. The concentrations at the extreme ends of the range should be examined carefully as these often have a large impact on the slope and calculations derived from the slope. If, in the final assay, the intent is to use only concentrations in the linear range, choose a range of concentrations that will yield parallel straight lines for the relative potencies expected during routine use of the assay; otherwise, the assay will fail parallelism tests when the potency produces assay response values outside the linear range of response. When potency is outside the linear range, it may be appropriate to adjust the sample concentration based on this estimated potency and test again in order to obtain a valid potency result. The repeat assays together with the valid assays may generate a biased estimate of potency because of the selective process of repeating assays when the response is in the extremes of the concentration–response curve.

The problem is more complex in assays where there is even modest variation in the shape or location of the concentration–response curve from run to run or from block to block within an assay. In such assays it may be appropriate to choose subsets for each sample in each assay or even in each block within an assay. Note that a fixed-effects model will mask any need for different subsets in different blocks, but a mixed-effects model may reveal and accommodate different subsets in different blocks (see section 4.9 Fixed and Random Effects in Models of Bioassay Response).

Additional guidance about selection of data subset(s) for linear model estimation of relative potency includes the following: use at least three, and preferably four, adjacent concentrations; require that the slope of the linear segment is sufficiently steep; require that the lines fit to Standard and Test samples are straight; and require that the fit regression lines are parallel. One way to derive a steepness criterion is to compute a t-statistic on the slope difference from zero. If the slope is not significant the bioassay is likely to have poor performance; this may be observed as increased variation in the potency results. Another aspect that supports requiring adequate steepness of slope is the use of subset selection algorithms. Without a slope steepness criterion, a subset selection algorithm that seeks to identify subsets of three or more contiguous data points that are straight and parallel might select concentrations on an asymptote. Such subsets are obviously inappropriate to use for potency estimation. How steep or how significant the steepness of the slope should be depends on the assay. This criterion should be set during assay development and possibly refined during assay validation.

4.6 Common Bioassay Models

Most bioassays consist of a series of concentrations or dilutions of both a Test sample and a Standard material. A mathematical model is fit to the concentration–response data, and a relative potency may then be calculated from the parameters of the model. Choice of model may depend on whether quantitative or qualitative data are being analyzed.

For quantitative data, models using parallel response profiles which support comparative evaluation for determining relative potency may provide statistical advantages. If such a model is used, concentrations or dilutions are usually scaled geometrically, e.g., usually in two-fold, log, or half-log increments. If a slope-ratio model is used, concentrations or dilutions can be equally spaced on concentration, rather than log concentration. Several functions may be used for fitting a parallel response model to quantitative data, including a linear function, a higher-order polynomial function, a four-parameter logistic (symmetric sigmoid) function, and a five-parameter logistic function for asymmetric sigmoidal responses. Such functions require a sufficient number of concentrations or dilutions to fit the model. To assess lack of fit of any model it is necessary to have at least one, and preferably several, more concentrations (or dilutions) than the number of parameters that will be estimated in the model. Also, at least one, and better, two, concentrations are commonly used to support each asymptote.

A linear model is sometimes selected because of apparent efficiency and ease of processing. Because bioassay response profiles are usually nonlinear, the laboratory might perform an experiment with a wide range of concentrations in order to identify the approximately linear region of the concentration–response profile. For data that follow a four-parameter logistic model, these are the concentrations near the center of the response region, often from 20% to 80% response when the data are rescaled to the asymptotes. Caution is appropriate in using a linear model because for a variety of reasons the apparently linear region may shift. A stable linear region may be identified after sufficient experience with the assay and with the variety of samples that are expected to be tested in the assay. Data following the four-parameter logistic function may also be linearized by transformation. The lower region of the function is approximately linear when the data are log transformed (log–log fit).

Quantal data are typically fit using more complex mathematical models. A probit or logit model may be used to estimate a percentile of the response curve (usually the 50th percentile) or, more directly, the relative potency of the Test to the Standard.
Spearman-Kärber analysis is a non-modeling method that may be employed for determining the 50th percentile of a quantal concentration–response curve.

### 4.7 Suitability Testing

System suitability and sample suitability assessment should be performed to ensure the quality of bioassay results. System suitability in bioassay, as in other analytical methods, consists of pre-specified criteria by which the validity of an assay (or, perhaps, a run containing several assays) is assessed. Analysts may assess system suitability by determining that some of the parameters of the Standard response are in their usual ranges and that some properties (e.g., residual variation) of the data are in their usual range. To achieve high assay acceptance rates, it is advisable to accept large fractions of these usual ranges (99% or more) and to assess system suitability using only a few uncorrelated Standard response parameters. The choice of system suitability parameters and their ranges may also be informed by empirical or simulation studies that measure the influence of changes in a parameter on potency estimation.

Sample suitability in bioassay is evaluated using pre-specified criteria for the validity of the potency estimate of an individual Test sample, and usually focuses on similarity assessment. System and sample suitability criteria should be established during bioassay development and before bioassay validation. Where there is limited experience with the bioassay, these criteria may be considered provisional.

#### SYSTEM SUITABILITY

System suitability parameters may be selected based on the design and the statistical model. Regardless of the design and model, however, system suitability parameters should be directly related to the quality of the bioassay. These parameters are generally based on standard and control samples. In parallel-line assays, for example, low values of the Standard slope typically yield estimates of potency with low precision. Rather than reject assays with low slope, analysts may find it more effective to use additional replicate assays until the assay system can be improved to consistently yield higher-precision estimates of potency. It may be particularly relevant to monitor the range of response levels and location of asymptotes associated with controls or Standard sample to establish appropriate levels of response. A drift or a trend in some of the criteria may indicate the degradation of a critical reagent or Standard material. Statistical process control (SPC) methods should be implemented to detect trends in system suitability parameters.

Two common measures of system suitability are assessment of the adequacy of the model (goodness of fit) and of precision. With replicates in a completely randomized design, a pure error term may be separated from the assessment of lack of fit. Care should be taken in deriving a criterion for lack of fit; the use of the wrong error term may result in an artificial assessment. The lack of fit sum of squares from the model fit to the Standard may, depending on the concentrations used and the way in which the data differ from the model, be a useful measure of model adequacy. A threshold may be established, based on sensitivity analysis (assessment of assay sensitivity to changes in the analyte) and/or historical data, beyond which the lack of fit value indicates that the data are not suitable. Note that the Test data are not used here; adequacy of the model for the Test is part of sample suitability.

For assessment of precision, two alternatives may be considered. One approach uses the mean squared error (residual variance) from the model fit to the Standard alone. Because this approach may have few degrees of freedom for the variance estimate, it may be more useful to use a pooled mean squared error from separate model fits to Standard and Test. Once the measure is selected, use historical data and sensitivity analysis to determine a threshold for acceptance.

#### SAMPLE SUITABILITY

Sample suitability in bioassay generally consists of the assessment of similarity, which can only be done within the assay range. Relative potency may be reported only from samples that both show similarity to Standard, exhibit requisite quality of model fit, and have been diluted to yield an EC\textsubscript{50} (and potency) within the range of the assay system.

#### SIMILARITY

In the context of similarity assessment, classical hypothesis (difference) testing evaluates a null hypothesis that a measure (a nonsimilarity parameter measuring the difference between Standard and Test concentration–response curves) is zero, with an implicit alternative hypothesis that the measure is non-zero or the statistical assumptions are not satisfied. The usual (“difference test”) criterion that the p-value must be larger than a certain critical value in order to declare the sample similar to reference controls the probability that samples are falsely declared nonsimilar; this is the producer’s risk of failing good samples. The consumer’s risk (the risk that nonsimilar samples are declared similar) is controlled via the precision in the nonsimilarity measure and amount of replication in the assay; typically these are poorly assessed, leaving consumer risk uncontrolled.

In contrast to difference testing, equivalence testing for similarity (assessing whether a 90% confidence interval for a measure of nonsimilarity is contained within specified equivalence bounds) allows only a 5% probability that samples with nonsimilarity measures outside the equivalence boundaries will be declared similar (controlling the consumer’s risk). With equivalence testing it is practical to examine and manage the producer’s risk by ensuring that there is enough replication in the assay to have good precision in estimating the nonsimilarity measure(s).

For the comparison of slopes, difference tests have traditionally been used to establish parallelism between a Test sample and the Standard sample. Using this approach the laboratory cannot conclude that the slopes are equal. The data may be too variable, or the assay design may be too weak to establish a difference. The laboratory can, however, conclude that the slopes are sufficiently similar using the equivalence testing approach.

Equivalence testing has practical advantages compared to difference testing, including that increased replication yielding improved assay precision will increase the chances that samples will pass the similarity criteria; that decreased assay replication or precision will decrease the chances that samples will pass the similarity criteria; and that sound approaches to combining
data from multiple assays of the same sample to better understand whether a sample is truly similar to Standard or not are obtained. Because of the advantages associated with the use of equivalence testing in the assessment of similarity, analysts may transition existing assays to equivalence testing or may implement equivalence testing methods when changes are made to existing assays. In this effort, it is informative to examine the risk that the assay will fail good samples. This risk depends on the precision of the assay system, the replication strategy in the assay system, and the critical values of the similarity parameters (this constitutes a process capability analysis). One approach to transitioning an established assay from difference testing to equivalence testing (for similarity) is to use the process capability of the assay to set critical values for similarity parameters. This approach is reasonable for an established assay because the risks (of falsely declaring samples similar and falsely declaring samples nonsimilar) are implicitly acceptable, given the assay’s history of successful use.

Similarity measures may be based on the parameters of the concentration–response curve and may include the slope for a straight parallel-line assay; intercept for a slope-ratio assay; the slope and asymptotes for a four-parameter logistic parallel-line assay; or the slope, asymptotes, and nonsymmetry parameter in a five-parameter sigmoid model. In some cases, these similarity measures have interpretable, practical meaning in the assay; certain changes in curve shape, for example, may be associated with specific changes (e.g., the presence of a specific active contaminant) in the product. When possible, discussion of these changes and their likely effects is a valuable part of setting appropriate equivalence boundaries.

IMPLEMENTING EQUIVALENCE TESTING FOR SIMILARITY (PARALLELISM)

As previously stated, many statistical procedures for assessing similarity are based on a null hypothesis stating that similarity is present and the alternative hypothesis of there being a state of nonsimilarity. Failure to find that similarity is statistically improbable is then taken as a conclusion of similarity. In fact, however, this failure to establish a probabilistic basis for nonsimilarity does not prove similarity. Equivalence testing provides a method for the analyst to proceed to a conclusion (if warranted by the data) of sufficiently similar while controlling the risk of doing so inappropriately. The following provides a sequence for this process of implementing equivalence testing.

Step 1: Choose a measure of nonsimilarity.

For the parallel-line case, this could be the difference or ratio of slopes. (The ratio of slopes can be less sensitive to the value of the slope. Framing the slope difference as a proportional change from Standard rather than in absolute slope units has an advantage because it is invariant to the units on the concentration and response axes.) For a slope-ratio assay, the measure of nonsimilarity can be the difference in y-intercepts between Test and Standard samples. Again, it can be advantageous to frame this difference as a proportion of the (possibly transformed) response range of Standard to make the measure invariant to the units of the response.

The determination of similarity could be based on the individual parameters, one at a time; for the four-parameter logistic model, similarity between Standard and Test samples can be assessed discretely for the upper asymptote, the slope, and the lower asymptote. If sigmoid curves with additional parameters are used to fit bioassay data, it is also important to consider addressing similarity between Standard and Test preparations of the additional curve parameters (e.g., asymmetry parameter of the five-parameter model). Alternatively, evaluation of similarity can be based on a single composite measure of nonparallelism, such as the parallelism sum of squares. This is found as the difference in residual sum of squared errors (RSSE) between the value obtained from fitting the Standard and Test curves separately and the value obtained from imposing parallelism:

\[
\text{Parallelism sum of squares} = \text{RSSE}_p - \text{RSSE}_T - \text{RSSE}_S,
\]

where the subscripts P, S, and T denote Parallel model, Standard model, and Test model, respectively. With any composite measure, the analyst must consider the implicit relative weighting of the importance of the three (or more) curve regions and whether the weighting is appropriate for the problem at hand. For the parallelism sum of squares, for example, with nonlinear models, the weighting given to the comparison of the asymptotes depends on the amount of data in the current assay on and near the asymptotes.

Step 2: Specify a range of acceptable values, typically termed an equivalence interval or “indifference zone,” for the measure of nonsimilarity.

The challenge in implementing equivalence testing is in setting appropriate equivalence bounds for the nonsimilarity measures. Ideally, information is available to link variation in similarity measures to meaningful differences in biological function (as measured by the bioassay). Information may be available from evaluation of orthogonal assays. The following four approaches can be used to determine this interval. If pharmacopeial limits have been specified for a defined measure of nonsimilarity, then the assay should satisfy those requirements.

a. The first approach is to compile historical data that compare the Standard to itself and using these data to determine the equivalence interval as a tolerance interval for the measure of nonparallelism. The advantage of using historical data is that they give the laboratory control of the false failure rate (the rate of failing a sample that is in fact acceptable). The disadvantage is that there is no control of the false pass rate (the rate of passing a sample that may have an unacceptable difference in performance relative to the Standard). The equivalence interval specification developed in this way is based solely on assay capability. Laboratories that use this approach should take caution that an imprecise assay in need of improvement may yield such a wide equivalence interval that no useful discrimination of nonsimilarity is possible.

b. Approach (a) is simple to implement in routine use and can be used with assay designs that do not provide reliable estimates of within-assay variation and hence confidence intervals. However, there is a risk that assays with larger than usual amounts of within-assay variation can pass inappropriately. The preferable alternative to (a) is therefore to determine a tolerance interval for the confidence interval for the measure of nonparallelism. The following is particularly appropriate to transition an existing assay with a substantial body of historical data on both Standard and Test samples from a difference testing approach to an equivalence approach.
i. For each value of the measure of nonparallelism from the historical data, determine a 95% confidence interval, $(m, n)$.

ii. For each confidence interval, determine its maximum departure from perfect parallelism. This is $\max(|m|, |n|)$ for differences, $\max(1/m, n)$ for ratios, and simply $n$ for quantities that must be positive, such as a sum of squares.

iii. Determine a tolerance interval for the maximum departures obtained in (ii). This will be a one-sided tolerance interval for these necessarily positive quantities. A nonparametric tolerance interval approach is preferred.

iv. “Sufficiently parallel” is concluded for new data if the confidence interval for the measure of nonparallelism falls completely within the interval determined in (iii).

Approaches (a) and (b), through their reliance on assay capability, control only the false fail rate, and neglect the false pass rate. Incorporating information from sources other than the evaluation of assay capability provides control of the false pass rate. Approaches (c) and (d) are means to this end.

c. The third approach starts with historical data comparing the Standard to itself and adds data comparing the Standard to known failures, e.g., to degraded samples. Compare values of the measure of nonsimilarity for data for which a conclusion of similarity is appropriate (Standard against itself) and data for which a conclusion of similarity is not appropriate, e.g., degraded samples. Based on this comparison, determine a value of the measure of nonsimilarity that discriminates between the two cases. If this approach is employed, a range of samples for which a conclusion of similarity is not appropriate should be utilized, including samples with the minimal important nonsimilarity. For nonlinear models, this comparison also can be used to determine which parameters should be assessed; some may not be sensitive to the failures that can occur with the specific assay or collection of nonsimilar samples.

d. The fourth approach is based on combining a sensitivity analysis of the assay curve to nonsimilarity parameters with what is known about the product and the assay. It is particularly helpful if information is available that links a shift in one or more nonsimilarity measures to properties of the product. These measures may be direct (e.g., changes in potency of a protein) or indirect (e.g., changes in efficacy or safety in an animal model). A complementary approach is provided by a limited sensitivity analysis that combines analyst and biologist judgment regarding the magnitude of shifts in a nonsimilarity parameter that are meaningful, with simulation and/or laboratory experiments, to demonstrate thresholds for similarity parameters that provide protection against important nonsimilarity. Additionally, risk analysis may be informed by the therapeutic index of the drug.

**Step 3. Examine whether the value of the nonsimilarity measure is found within the equivalence interval of acceptable values.**

For approaches (a) and (b), compare the obtained value of the measure of nonparallelism (a) or its confidence interval (b) to the interval obtained at the beginning of Step 2. The value must be within the limits if one uses (a), or the confidence interval must be completely within the limits if one uses (b).

An alternative to the approach described above [for (a)] is to use an average (historical) value for the variance of the ratio or difference in a similarity parameter—obtained from some number of individual assays—to compute an acceptance interval for a point estimate of the similarity parameter. This approach is simpler to implement in routine use and can be used with assay designs that are unable to provide reliable estimates of within-assay variation. However, there is a price. The equivalence testing approach that relies on assay-specific (within-assay) measure(s) of variation (i.e., the confidence intervals) is conservative in the sense that it will fail to pass similarity for samples from assays that have larger than usual amounts of within-assay variation. Using an acceptance region for a similarity parameter—rather than an acceptance region for confidence intervals for the similarity parameter—loses this conservative property and hence is not preferred where alternatives exist.

For approach (c), an approach that essentially treats the parallelism as a discrimination problem may be used. The choice of the cut point in (c) should take into account the rates of false positive and false negative decisions (and the acceptable risks to

For approach (d), demonstrate that the measure of nonsimilarity is significantly greater than the lower endpoint of the acceptance interval and significantly less than the upper endpoint. (If the acceptance interval is one-sided, then apply only the single applicable test.) This is use of the TOST approach. For most situations, TOST can be most simply implemented by calculating a 90% two-sided confidence interval, which corresponds to a 5% equivalence test. If this confidence interval lies entirely within the equivalence interval specified at the beginning of Step 2, then similarity is sufficiently demonstrated. For parallel-line models, one can use either (1) a confidence interval based on the value of the difference of the slopes $\pm k$ times the standard error of that value, or (2) Fieller’s Theorem for the ratio of slopes may be used. For slope ratio models use the confidence interval for the difference of intercepts. For nonlinear models, there is evidence that these simple confidence interval methods do not attain the stated level of confidence, and methods based on likelihood profile or resampling are more appropriate.

**RANGE**

The range for a relative potency bioassay is the interval between the upper and lower relative potencies for which the bioassay is shown to have suitable levels of precision, relative accuracy, linearity of log potency, and success rates for system and sample suitability. It is straightforward to determine whether or not a sample that is similar to a Standard has a relative potency within the (validated) range of the assay system. For samples that are not similar according to established criteria, it is more challenging to determine whether a relative potency estimate for the sample might be obtained. In a nonlinear parallel-line assay a sample that does not have data on one asymptote might be assumed to be out of the potency range of the assay. In a parallel straight-line assay a sample that does not have three or more points on the steep portion of the response curve may be out of the potency range of the assay. For samples that have not been shown to be similar to reference it is not appropriate to report potency or to construct a ratio of $EC_{50}$ from unrestricted fits. As such samples may be out of the assay range, it may be useful to shift the dilution of the test sample for a subsequent assay on the basis of an estimate of relative activity. This estimated relative activity may be obtained via the ratio of the concentrations of Standard and Test that yields responses that match the reference response at the reference $EC_{50}$. 

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4.8 Outliers

Bioassay data should be screened for outliers before relative potency analysis. Outliers may be simple random events or a signal of a systematic problem in the bioassay. Systematic error that generates outliers may be due to a dilution error at one or more concentrations of a Test sample or the Standard or due to a mechanical error (e.g., system malfunction). Several approaches for outlier detection can be considered. Visual inspection is frequently utilized but should be augmented with a more objective approach to avoid potential bias.

An outlier is a datum that appears not to belong among the other data present. An outlier may have a distinct, identifiable cause, such as a mistake in the bench work, equipment malfunction, or a data recording error, or it could just be an unusual value relative to the variability typically seen and may appear without an identifiable cause. The essential question pertaining to an outlier becomes: Is the apparent outlier sampled from the same population as the other, less discordant, data, or is it from another population? If it comes from the same population and the datum is, therefore, an unusual (yet still legitimate) value obtained by chance, then the datum should stand. If it comes from another population and the datum’s exclusive value is due to human error or instrument malfunction, then the datum should be omitted from calculations. In practice, the answer to this essential question is often unknown, and investigations into causes are often inconclusive. Outlier management relies on procedures and practices to yield the best answer possible to that essential question and to guide response accordingly.

General chapter Analytical Data—Interpretation and Treatment (1010) addresses outlier labeling, identification, and rejection; statistical methods are included. General chapter (1010) also lists additional sources of information that can provide a comprehensive review of the relevant statistical methodology. General chapter (1010) makes no explicit remarks regarding outlier analysis in linear or nonlinear regression. Outlier analysis techniques appropriate for data obtained from regression of response on concentration can be used. Some remarks about outliers are provided here in the context of bioassays to emphasize or complement the information in (1010).

Outlier prevention is preferred for obvious reasons, and is facilitated by procedures that are less subject to error and by checks that are sensitive to the sorts of errors that, given the experience gained in assay development, may be expected to occur. In effect, the error never becomes an outlier because it is prevented from occurring.

Good practice calls for the examination of data for outliers and labeling (“flagging”) of the apparently outlying observation(s) for investigation. If investigation finds a cause, then the outlier and datum may be excluded from analysis. Because of the ordinary occurrence of substantial variability in bioassay response, a laboratory’s investigation into the outlying observation is likely to yield no determinable cause. However, the lack of evidence regarding an outlier’s cause is not a clear indication that statistical outlier testing is warranted. Knowledge of the typical range of assay response variability should be the justification for the use of statistical outlier tests.

Outlier identification is the use of rules to confirm that the values are inconsistent with the known or assumed statistical model. For outliers with no determined cause, it is tempting to use statistical outlier identification procedures to discard unusual values. Discarding data solely because of statistical considerations should be a rare event. Falsely discarding data leads to overly optimistic estimates of variability and can bias potency estimates. The laboratory should monitor the failure rate for its outlier procedure and should take action when this is significantly higher than expected.

Statistical procedures for outlier identification depend on assumptions about the distribution of the data without outliers. Identification of data as outliers may mean only that the assumption about distribution is not correct. If dropping outliers because of statistical considerations is common, particularly if outliers tend to occur more often at high values or at high responses, then this may be an indication that the data require some adjustment, such as log transformation, as part of the assay procedure. Two approaches to statistical assessment of outlying data are replication-based and model-based.

REPLICATION-BASED APPROACHES

When replicates are performed at concentrations of a Test sample and the Standard, an “extra variability” (EV) criterion may be employed to detect outliers. Historical data can be analyzed to determine the range in variability commonly observed among replicates, and this distribution of ranges can be used to establish an extreme in the range that might signal an outlier. Metrics that can be utilized are the simple range (maximum replicate minus minimum replicate), the standard deviation, or the CV or RSD among replicates. However, if the bioassay exhibits heterogeneity of variability, assumptions about uniform scatter of data are unsupported. Analysts can use a variable criterion across levels in the bioassay, or they can perform a transformation of the data to a scale that yields homogeneity of variability. Transformation can be performed with a POM approach as discussed previously. Where heterogeneity exists nonnormality is likely present, and the range rather than standard deviation or RSD should be used.

The actions taken upon detection of a potential outlier depend in part on the number of replicates. If EV is detected within a pair (n = 2) at a concentration of a Test sample or the Standard, it will not always be clear which of the replicates is aberrant, and the laboratory should eliminate the concentration from further processing. If more than two replicates are performed at each dilution the laboratory may choose to adopt a strategy that identifies which of the extremes may be the outlier. Alternatively, the laboratory may choose to eliminate the dilution from further processing.

MODEL-BASED APPROACHES

Model-based approaches may be used to detect outliers within bioassay data. These approaches use the residuals from the fit of an appropriate model. In general, if using model-based methods to identify potential outliers, the models used may make
fewer assumptions about the data than the models used to assess suitability and estimate potency. For example, a non-parametric regression (smoothing) model may be useful.

Lastly, an alternative to discarding outlying data is to use robust methods that are less sensitive to influence by outlying observations. Use of the median rather than the mean to describe the data’s center exemplifies a robust perspective. Also, regression using the method of least squares, which underlies many of the methods in this chapter, is not robust in the presence of outliers. The use of methods such as robust regression may be appropriate but is not covered in the USP bioassay chapters.

### 4.9 Fixed and Random Effects in Models of Bioassay Response

The choice of treating factors as fixed or random is important for the bioassay design, the development experiments, the statistical analysis of data, and the bioassay validation. Fixed effects are factors for which all levels, or all levels of interest, are discretely present, like sample, concentration, temperature and duration of thaw, and incubation time. Data for a response at some level, or combination of levels, of a fixed factor, can predict future responses. Fixed effects are expected to cause a consistent shift in responses. Analysts study fixed effects by controlling them in the design and examining changes in means across levels of the factor.

Random effects are factors of which the levels in a particular run of an assay are considered representative of levels that could be present. That is, there is no expectation that any specific value of the random factor will influence response. Rather, that value may vary subject to some expected distribution of values and thus may be a source of variability. For example, there is no desire to predict assay response for a specific day, but there is interest in predicting the variation in response associated with the factor “day”. Examples of random effects include reagent lot, operator, or day if there is no interest in specific reagent lots, operators, or day as sources of variability. Analysts may study random effects by measuring the variance components corresponding to each random effect. Variance components can be estimated well only if there are an appreciable number of levels of each random effect. If there are, for example, only two or three reagent lots or analysts present, the variation associated with these factors will be poorly estimated.

Making a correct choice regarding treating a factor as fixed or random is important to the design of the assay and to proper reporting of its precision. Treating all factors as fixed, for example, leads to an understatement of assay variability because it ignores all sources of variability other than replication. The goal is to identify specific sources of variability that can be controlled, to properly include those factors in the design, and then to include other factors as random.

If the factor may switch from random to fixed effect or vice versa, the factor should normally be modeled as a random effect. For example, reagent lots cannot be controlled, so different lots are typically considered to cause variability, and reagent lot would be considered a random effect. However, if a large shift in response values has been traced to a particular lot, a comparison among a set of lots could be performed using reagent lot as a fixed effect. Similarly, within-assay location (e.g., block, plate, plate row, plate column, or well) or sequence may be considered a source of random variation or a source of a consistent (fixed) effect.

Assay designs that consist of multiple factors are efficient, but require corresponding statistical techniques that incorporate the factors as fixed or random effects in the analysis. If all factors are fixed, the statistical model is termed a fixed-effects model. If all are random, it is termed a random-effects model. If some factors are fixed and some random, the model is a mixed-effects model. Note that the concepts of fixed and random effects apply to models for quantitative, qualitative and integer responses. For assay designs that include multiple experimental units (e.g., samples assigned to sets of tubes and concentrations assigned to pre-plate tubes) a mixed-effects model in which the experimental units are treated as random effects is particularly effective. Additional complexity is added by the presence of designs with crossed random effects (e.g., each operator used material from one or more reagent batches, but many reagent batches were used by multiple operators). This can cause methodological and computational challenges for model fitting, especially when the designs are unbalanced.

### 5. Stages in the Bioassay Development Process

Given the ubiquity of cell-based assays and the motivation to use one bioassay system to provide context for discussion, the development of a cell-based bioassay will be used to illustrate the stages in the bioassay development continuum.

#### 5.1 Design: Assay Layout, Blocking, and Randomization

Most cell-based assays are performed using a cell culture plate (6-, 12-, 96-, or 384-well micro titer plate). Ideally, a plate is able to provide a uniform substrate for experimental treatments in all wells, including after wash steps and incubations. However, regardless of assay conditions intended to minimize the potential for bias (e.g., good analyst technique, careful calibration of pipets, controlled incubation time, and temperature), systematic gradients on the plate, independent of experimental treatments, may be observed. These gradients may occur across rows, across columns, or from the edge to the center of the plate and are often called plate effects. Even moderate or inconsistent plate effects should be addressed during assay development, by means of plate layout strategies, blocking, randomization, and replication.

Plate effects can be evaluated in a uniformity trial in which a single experimental treatment, such as an assay concentration chosen from the middle section of the concentration–response curve, is used in all wells of the plate. Figure 1 provides an example of what may be observed; a trend of decreasing signal is evident from right to left. In this case, it was discovered that the plate washer was washing more briskly on the left side of the plate, and required adjustment to provide uniform washing intensity and eliminate the gradient. Another common plate effect is a differential cell-growth pattern in which the outer wells of the plate grow cells in such a way that the assay signal is attenuated. This is such a persistent problem that the choice is often made to not use the outer wells of the assay plate. Because location effects are so common, designs that place replicates (e.g., of sample by concentration combinations) in adjacent wells should be avoided.
Blocking is the grouping of related experimental units in experimental designs. Blocks may consist of individual 96-well plates, sections of 96-well plates, or 96-well plates grouped by analyst, day, or batch of cells. The goal is to isolate any systematic effects so that they do not obscure the effects of interest. A complete block design occurs when all levels of a treatment factor (in a bioassay, the primary treatment factors are sample and concentration) are applied to experimental units for that factor within a single block. An incomplete block design occurs when the number of levels of a treatment factor exceeds the number of experimental units for that factor within the block.

Randomization is a process of assignment of treatment to experimental units based on chance so that all such experimental units have an equal chance of receiving a given treatment. Although challenging in practice, randomization of experimental treatments has been advocated as the best approach to minimizing assay bias or, more accurately, to protecting the assay results from known and unknown sources of bias by converting bias into variance. While randomization of samples and concentrations to individual plate wells may not be practical, a plate layout can be designed to minimize plate effects by alternating sample positions across plates and the pattern of dilutions within and across plates. Where multiple plates are required in an assay, the plate layout design should, at a minimum, alternate sample positions across plates within an assay run to accommodate possible bias introduced by the analyst or equipment on a given day. It is prudent to use a balanced rotation of layouts on plates so that the collection of replicates (each of which uses a different layout) provides some protection against likely sources of bias.

Figure 2 illustrates a patterned assay design that lacks randomization and is susceptible to bias. Dilutions and replicates of the Test preparations (A and B) and the Standard (R) are placed together sequentially on the plate. Bias due to a plate or incubator effect can influence some or all of the concentrations of one of the samples. Note that in Figures 2 through 5 all outer plate wells are left as blanks to protect against edge effect.
A layout that provides some protection from plate effects and can be performed manually is a **strip-plot design**, shown in Figure 3. Here samples are randomized to rows of a plate and dilution series are performed in different directions in different sections (blocks) on the plate to mitigate bias across columns of the plate. An added advantage of the strip-plot design is the ability to detect location effects by the interaction of sample and dilution direction (left-to-right or right-to-left).

![Figure 3. A strip-plot design.](image)

**Figure 3. A strip-plot design.**

**Figure 4** illustrates an alternation of Test (Test sample 1 = “1”; Test sample 2 = “2”) and Standard (“R”) positions on multiple plates, within a single assay run; this protects against plate row effects. Combining the two methods illustrated if Figures 3 and 4 can effectively help convert plate bias into assay variance. Assay variance may then be addressed, as necessary, by increased assay replication (increased number of plates in an assay).

![Figure 4. A multi-plate assay with varied Test and Reference positions.](image)

**Figure 4. A multi-plate assay with varied Test and Reference positions.**

A **split-plot design**, an alternative that assigns samples to plate rows randomly and randomizes dilutions (concentrations) within each row, is seen in Figure 5. Such a strategy may be difficult to implement even with the use of robotics.
Assay concentrations of a Test sample and the Standard can be obtained in different ways. Laboratories often perform serial dilutions, in which each dilution is prepared from the previous one, in succession. Alternatively, the laboratory may prepare wholly independent dilutions from the Test sample and Standard to obtain independent concentration series. These two strategies result in the same nominal concentrations, but they have different properties related to error. Serial dilutions are subject to propagation of error across the dilution series, and a dilution error made at an early dilution will result in correlated, non-independent observations. Correlations may also be introduced by use of multichannel pipets. Independent dilutions help mitigate the bias resulting from dilution errors.

It is noteworthy that when working to improve precision, the biggest reductions in variance come when replicating at the highest possible levels of nested random effects. This is particularly effective when these highest levels are sources of variability. To illustrate: replicating extensively within a day for an assay known to have great day-to-day variation is not effective in improving precision of reportable values.

5.2 Development

A goal of bioassay development is to achieve optimal bioassay relative accuracy and precision of the potency estimate. An endpoint of assay development is the completed development of the assay procedure, a protocol for the performance of the bioassay. The procedure should include enough detail so that a qualified laboratory with a trained analyst can perform the procedure in a routine manner. A strategic part of development is a look forward toward performance maintenance. Standard operating procedures for reagent and technician qualification, as well as for calibration of the working Standard, help complete the bioassay development package.

ONE FACTOR AT A TIME VERSUS DESIGN OF EXPERIMENTS

Bioassay development proceeds through a series of experiments in which conditions and levels of assay factors are varied to identify those that support a reliable and robust bioassay qualified for routine use. Those experiments may be conducted one factor at a time (OFAT), studying each parameter separately to identify ideal conditions, or through the use of multi-factor design of experiments (DOE). DOE is an efficient and effective strategy for developing a bioassay and improving bioassay performance, thus helping to obtain a measurement system that meets its requirements. In comparison to OFAT, DOE generally requires fewer experiments and also provides insight into interactions of factors that affect bioassay performance. Assay development using DOE may proceed through a series of steps: process mapping and risk analysis; screening; response optimization; and confirmation.

PROCESS MAPPING AND RISK ANALYSIS

Bioassay optimization may begin with a systematic examination and risk assessment to identify those factors that may influence bioassay response. It is useful to visualize bioassay factors using a bioassay process map such as a cause-and-effect or fishbone diagram. Using the process map as a guide, the laboratory can examine assay factors that might affect assay performance, such as buffer pH, incubation temperature, and incubation time. Historical experience with one or several of the bioassay steps, along with sound scientific judgment, can identify key factors that require further evaluation. One tool that may be used to prioritize factors is a failure mode and effects analysis. Factors are typically scored by the combination of their potential to influence assay response and the likelihood that they will occur. The laboratory must be careful to recognize potential interactions between assay factors.
SCREENING

Once potential key factors have been identified from process mapping and risk analysis, the laboratory may conduct an initial screening experiment to probe for effects that may require control. Screening designs such as factorial and fractional factorial designs are commonly used for this purpose. Software is available to assist the practitioner in the selection of the design and in subsequent analysis. Analysts should take care, however, to understand their assumptions about design selection and analysis to ensure accurate identification of experimental factors.

RESPONSE OPTIMIZATION

A screening design will usually detect a few important factors from among those studied. Such factors can be further studied in a response-optimization design. Response-optimization designs such as central composite designs are performed to determine optimal settings for combinations of bioassay factors for achieving desired response. The information obtained from response optimization may be depicted as a response surface and can be used to establish ranges that yield acceptable assay performance and will be incorporated into the bioassay procedure.

In the parlance of Quality by Design (QbD), the “region” where the combined levels of input variables and process parameters have been demonstrated to provide acceptable assay performance is described as the design space for the bioassay. Establishing a true design space for a bioassay is challenging; some but not all factors and levels of random factors will be included in the development DOE, and there is no assurance that the design space is not sensitive to unstudied random factors. Similarly, there is little assurance that the assay (design space) is robust to random factors that are studied using small samples (or non-random samples of levels). Elements of DOE that may be considered include the use of blocks; deliberate confounding among interactions that are of lower interest, or known to be unimportant; robust design (response surface designs with random effects); and use of split-plot, strip-plot, or split-lot designs.

CONFIRMATION

The mathematical model depicting assay performance as a function of changes in key assay factors is an approximation; thus, it is customary to confirm performance at the ideal settings of the bioassay. Confirmation can take the form of a qualification trial in which the assay is performed, preferably multiple independent times using optimal values for factors. Alternatively, the laboratory may determine that the bioassay has been adequately developed and may move to validation. Qualification is a good practice, not a regulatory requirement. The decision to perform confirmatory qualifying runs or to proceed to validation depends upon the strength of the accumulated information obtained throughout development.

5.3 Data Analysis during Assay Development

Analysis of bioassay data during assay development enables analysts to make decisions regarding the statistical model that will be used for routine analysis, including transformation and/or weighting of data, and the development of system and sample suitability criteria. The analysis also provides information regarding which elements of design structure should be used during outlier detection and the fitting of a full model. This may also include a plan for choosing subsets of data, such as a linear portion, for analysis or, for nonlinear bioassays, a model reduction strategy for samples similar to Standard. Once these decisions are made and proven sound during validation, they don’t need to be reassessed with each performance of the assay. A process approach to enabling these decisions follows.

Step 1: Choose an appropriate statistical model (also see section 4.6 Common Bioassay Models). Given the complexity of bioassays and the motivation to use an approach proven reliable, fairly standardized analytical models are common in the field of bioassay analysis. Nonetheless, many considerations are involved in choosing the most appropriate statistical model. First, the model should be appropriate for the type of assay endpoint—continuous, count, or dichotomous. Second, the model should incorporate the structure of the assay design. For any design other than completely randomized, there will be terms in the model for the structural elements. These could be, for example, within-plate blocking, location of cage in the animal facility, day, etc. A third consideration, applicable to continuous endpoints, involves whether to use a regression model or a means model (an analysis of variance model that fits a separate mean at each dilution level of each sample tested), with appropriate error terms. A means model can be appropriate at this stage because it makes no assumptions about the shape of the concentration–response curve.

Step 2: Fit the chosen statistical model to the data without the assumption of parallelism, and then assess the distribution of the residuals, specifically examining them for departures from normality and constant variance. Transform the data as necessary or, if needed, choose a weighting scheme (see section 4.3 Variance Heterogeneity, Weighting, and Transformation). Use as large a body of assay data, from independent assays, as possible. The primary goal is to address any departure from normality and from constant variance of responses across the range of concentrations in the assay. Step 2 will likely alternate between imposing a transformation and assessing the distribution of the residuals.

Step 3: Screen for outliers, and remove as is appropriate. This step normally follows the initial choice of a suitable transformation and/or weighting method. Ideally the model used for outlier detection contains the important elements of the assay design structure, allows nonsimilar curves, and makes fewer assumptions about the functional shape of the concentration–response curve than did the model used to assess similarity. See section 4.8 Outliers and general chapter (1010) for discussion of outlier detection and removal. In some cases, outliers may be so severe that a reasonable model cannot be fit, and thus residuals will not be available. In such cases, it is necessary to screen the raw data for outliers before attempting to fit the model.

During assay development, a strategy should be developed for the investigation and treatment of an outlier observation, including any limits on how many outliers are acceptable. Include these instructions in the assay SOP. Good practice includes recording the process of an investigation, outlier test(s) applied, and results therefrom. Note that outlier
procedures must be considered apart from the investigation and treatment of an out-of-specification (OOS) result (reportable value). Decisions to remove an outlier from data analysis should not be made on the basis of how the reportable value will be affected (e.g., a potential OOS result). Removing data as outliers should be rare. If many values from a run are removed as outliers, that run should be considered suspect.

Step 4: Refit the model with the transformation and/or weighting previously imposed (Step 2) without the observations identified as outliers (Step 3) and re-assess the appropriateness of the model.

Step 5: If necessary or desired, choose a scheme for identifying subsets of data to use for potency estimation, whether the model is linear or nonlinear (see section 4.5 Linearity of Concentration–Response Data).

Step 6: Calculate a relative potency estimate by analyzing the Test and Standard data together using a model constrained to have parallel lines or curves, or equal intercepts.

5.4 Bioassay Validation

The bioassay validation is a protocol-driven study that demonstrates that the procedure is fit for use. A stage-wise approach to validation may be considered, as in a “suitable for intended use” validation to support release of clinical trial material, and a final, comprehensive validation prior to BLA or MAA filing. Preliminary system and sample suitability controls should be established and clearly described in the assay procedure; these may be finalized based on additional experience gained in the validation exercise. Chapter 1033 provides validation comprehensive discussion of bioassay validation.

5.5 Bioassay Maintenance

The development and validation of a bioassay, though discrete operations, lead to ongoing activities. Assay improvements may be implemented as technologies change, as the laboratory becomes more skilled with the procedure, and as changes to bioassay methodology require re-evaluation of bioassay performance. Some of these changes may be responses to unexpected performance during routine processing. Corrective action should be monitored using routine control procedures. Substantial changes may require a study verifying that the bioassay remains fit for use. An equivalence testing approach can be used to show that the change has resulted in acceptable performance. A statistically-oriented study can be performed to demonstrate that the change does not compromise the previously acceptable performance characteristics of the assay.

ASSAY TRANSFER

Assay transfer assumes both a known intended use of the bioassay in the recipient lab and the associated required capability for the assay system. These implicitly, though perhaps not precisely, demarcate the limits on the amount of bias and loss of precision allowed between labs. Using two laboratories interchangeably to support one product will require considering the variation between labs in addition to intermediate precision for sample size requirements to determine process capability. For a discussion and example pertaining to the interrelationship of bias, process capability, and validation, see A Bioassay Validation Example in (1033).

IMPROVING OR UPDATEING A BIOASSAY SYSTEM

A new version of a bioassay may improve the quality of bias, precision, range, robustness, specificity, lower the operating costs or offer other compelling advantages. When improving or updating a bioassay system a bridging study may be used to compare the performance of the new to the established assay. A wide variety of samples (e.g., lot release, stability, stressed, critical isoforms) can be used for demonstrating equivalence of estimated potencies. Even though the assay systems may be quite different (e.g., an animal bioassay versus a cell-based bioassay), if the assays use the same Standard and mechanism of action, comparable potencies may reasonably be expected. If the new assay uses a different Standard, the minimum requirement for an acceptable comparison is a unit slope of the log linear relationship between the estimated potencies. An important implication of this recommendation is that poor precision or biased assays used early can have lasting impact on the replication requirements, even if the assay is later replaced by an improved assay.

(1033) BIOLOGICAL ASSAY VALIDATION

1. INTRODUCTION

Biological assays (also called bioassays) are an integral part of the quality assessment required for the manufacturing and marketing of many biological and some non-biological drug products. Bioassays commonly used for drug potency estimation can be distinguished from chemical tests by their reliance on a biological substrate (e.g., animals, living cells, or functional complexes of target receptors). Because of multiple operational and biological factors arising from this reliance on biology, they typically exhibit a greater variability than do chemically-based tests.

Bioassays are one of several physicochemical and biologic tests with procedures and acceptance criteria that control critical quality attributes of a biological drug product. As described in the ICH Guideline entitled Specifications: Test Procedures And Acceptance Criteria For Biotechnological/Biological Products (Q6B), section 2.1.2, bioassay techniques may measure an organism’s biological response to the product; a biochemical or physiological response at the cellular level; enzymatic reaction rates or biological responses induced by immunological interactions; or ligand- and receptor-binding. As new biological drug products and new technologies emerge, the scope of bioassay approaches is likely to expand. Therefore, general chapter
Biological Assay Validation (1033) emphasizes validation approaches that provide flexibility to adopt new bioassay methods, new biological drug products, or both in conjunction for the assessment of drug potency.

Good manufacturing practice requires that test methods used for assessing compliance of pharmaceutical products with quality requirements should meet appropriate standards for accuracy and reliability. Assay validation is the process of demonstrating and documenting that the performance characteristics of the procedure and its underlying method meet the requirements for the intended application and that the assay is thereby suitable for its intended use. USP general chapter Validation of Compendial Procedures (1225) and ICH Q2(R1) describe the assay performance characteristics (parameters) that should be evaluated for procedures supporting small-molecule pharmaceuticals. Although evaluation of these validation parameters is straightforward for many types of analytical procedures for well-characterized, chemically-based drug products, their interpretation and applicability for some types of bioassays has not been clearly delineated. This chapter addresses bioassay validation from the point of view of the measurement of activity rather than mass or other physicochemical measurements, with the purpose of aligning bioassay performance characteristics with uses of bioassays in practice.

Assessment of bioassay performance is a continuous process, but bioassay validation should be performed when development has been completed. Bioassay validation is guided by a validation protocol describing the goals and design of the validation study. General chapter (1033) provides validation goals pertaining to relative potency bioassays. Relative potency bioassays are based on a comparison of bioassay responses for a Test sample to those of a designated Standard that provides a quantitative measure of the Test bioactivity relative to that of the Standard.

Validation parameters discussed include relative accuracy, specificity, intermediate precision, and range. Laboratories may use dilutional linearity to verify the relative accuracy and range of the method. Although robustness is not a requirement for validation, general chapter (1033) recommends that a bioassay’s robustness be assessed prior to validation. In addition, (1033) describes approaches for validation robustness studies (which are usually performed during bioassay development, key factors in validation design). Validation acceptance criteria, data analysis and interpretation, and finally bioassay performance monitoring through quality control. Documentation of bioassay validation results is also discussed, with reference to pre-validation experiments performed to optimize bioassay performance. In the remainder of general chapter (1033) the term “bioassay” should be interpreted as meaning “relative potency bioassay”.

2. FUNDAMENTALS OF BIOASSAY VALIDATION

The goal of bioassay validation is to confirm that the operating characteristics of the procedure are such that the procedure is suitable for its intended use. The issues involved in developing a bioassay are described in greater detail in general chapter (1032) and are assumed resolved by the time the bioassay is in validation. Included in those decisions will be identification of what constitutes an assay and a run for the bioassay. Multiple dilutions (concentrations) of the Standard and one or more Test samples constitute a replicate set (also known as a minimal set), which contain a test substrate (e.g., group of animals or vessel of cells) at each dilution for each sample [Test(s) and Standard]. A run is defined as work performed during a period when the accuracy (trueness) and precision in the assay system can reasonably be expected to be stable. In practice, a run frequently consists of the work performed by a single analyst in one lab, with one set of equipment, in a short period of time (typically a day). An assay is the body of data used to assess similarity and estimate potency relative to a Standard for each Test sample in the assay. A run may contain multiple assays, a single assay, or part of an assay. Multiple assays may be combined to yield a reportable value for a sample. The reportable value is the value that is compared to a product specification.

In assays that involve groups at each dilution (e.g., 6 samples, each at 10 dilutions, in the non-edge wells of each of several 96-well cell culture plates) the groups (plates) constitute statistical blocks that should be elements in the assay and validation analyses (blocks are discussed in (1032)). Within-block replicates for Test samples are rarely cost-effective. Blocks will not be further discussed in this chapter; more detailed discussion is found in (1032).

The amount of activity (potency) of the Standard is initially assigned a value of 1.0 or 100%, and the potency of the Test sample is calculated by comparing the concentration–response curves for the Test and Standard pair. This results in a unitless measure, which is the relative potency of the Test sample in reference to the potency of the Standard. In some cases the Standard is assigned a value according to the potency property of interest (e.g., in a potency test). In that case the potency of the Test sample is the relative potency times the assigned value of the Standard. An assumption of parallel-line or parallel-curve (e.g., four-parameter logistic) bioassays is that the dose–response curves that are generated using a Standard and a Test sample have similar (parallel) curve shape distinguished only by a horizontal shift in the log dose. For slope-ratio bioassays, curves generated for Standard and Test samples should be linear, pass through a common intercept, and differ only by their slopes. Information about how to assess parallelism is provided in general chapters (1032) and (1034).

In order to establish the relative accuracy and range of the bioassay, validation Test samples may be constructed using a dilution series of the Standard to assess dilutional linearity (linearity of the relationship between known and measured relative potency). In addition, the validation study should yield a representative estimate of the variability of the relative potency. Determination of variability for validation results (e.g., studies) are usually performed during bioassay development, key factors in validation design of bioassays. Key factors in these studies such as incubation time and temperature and, for cell-based bioassays, cell passage number and cell number may be included in the validation, particularly if they interact with another factor that is introduced during the validation (e.g., a temperature sensitive reagent that varies in its sensitivity from lot-to-lot). Because of potential influences on the bioassay from inter-run factors such as multiple analysts, instruments, or reagent sources, the design of the bioassay validation should include consideration of these factors. The variability of potency from these combined elements defines the intermediate precision (IP) of the bioassay. An appropriate study of the variability of the potency values obtained, including the impact of intra-assay and inter-run factors, can help the laboratory confirm an adequate testing strategy and forecast the inherent variability of the reportable value (which may be the average of multiple potency determinations). Variability estimates can also be utilized to establish the sizes of differences (fold difference) that can be distinguished between samples tested in the bioassay. (See section 3.4 Use of Validation Results for Bioassay Characterization.)

Demonstrating specificity (also known as selectivity) requires evidence of lack of influence from matrix components such as manufacturing process components or degradation products so that measurements quantify the target molecule only. Other analytical methods may complement a bioassay in measuring or identifying other components in a sample.
2.1 Bioassay Validation Protocol

A bioassay validation protocol should include the number and types of samples that will be studied in the validation; the study design, including inter-run and intra-run factors; the replication strategy; the intended validation parameters and justified target acceptance criteria for each parameter; and a proposed data-analysis plan. Note that in regard to satisfying acceptance criteria, failure to find a statistically significant effect is not an appropriate basis for defining acceptable performance in a bioassay; conformance to acceptance criteria may be better evaluated using an equivalence approach.

In addition, assay, run, and sample acceptance criteria such as system suitability and similarity should be specified before performing the validation. Depending on the extent of development of the bioassay, these may be proposed as tentative and can be updated with data from the validation. Assay, run, or sample failures may be reassessed according to criteria which have been defined in the validation protocol and, with sound justification, included in the overall validation assessment. Additional validation trials may be required in order to support changes to the method.

The bioassay validation protocol should include target acceptance criteria for the proposed validation parameters. Steps to be taken upon failure to meet a target acceptance criterion should be specified in the validation protocol, and may result in a limit on the range of potencies that can be measured in the bioassay or a modification to the replication strategy in the bioassay procedure.

2.2 Documentation of Bioassay Validation Results

Bioassay validation results should be documented in a bioassay validation report. The validation report should support the conclusion that the method is fit for use or should indicate corrective action (such as an increase in the replication strategy) that will be undertaken to generate sufficiently reliable results to achieve fitness for use. The report could include the raw data and intermediate results (e.g., variance component estimates should be provided in addition to overall intermediate precision) which would facilitate reproduction of the bioassay validation analysis by an independent reviewer. Estimates of validation parameters should be reported at each level and overall as appropriate. Deviations from the validation protocol should be documented with justification. The conclusions from the study should be clearly described with references to follow-up action as necessary. Follow-up action can include amendment of system or sample suitability criteria or modification of the bioassay replication strategy. Reference to prevalidation experiments may be included as part of the validation study report. Prevalidation experiments may include robustness experiments, where bioassay parameters have been identified and ranges have been established for significant parameters, and also may include qualification experiments, where the final procedure has been performed to confirm satisfactory performance in routine operation. Conclusions from prevalidation and qualification experiments performed during development contribute to the description of the operating characteristics of the bioassay procedure.

2.3 Bioassay Validation Design

The biological assay validation should include samples that are representative of materials that will be tested in the bioassay and should effectively establish the performance characteristics of the procedure. For relative accuracy, sample relative potency levels that bracket the range of potencies that may be tested in the bioassay should be used. Thus samples that span a wide range of potencies might be studied for a drug or biological with a wide specification range or for a product that is inherently unstable, but a narrower range can be used for a more durable product. A minimum of three potency levels is required, and five are recommended for a reliable assessment. If the validation criteria for relative accuracy and IP are satisfied, the potency levels chosen will constitute the range of the bioassay. A limited range will result from levels that fail to meet their target acceptance criteria. Samples may also be generated for the bioassay validation by stressing a sample to a level that might be observed in routine practice (i.e., stability investigations). Additionally, the influences of the sample matrix (excipients, process constituents, or combination components) can be studied strategically by intentionally varying these together with the target analyte, using a multifactorial approach. Often this will have been done during development, prior to generating release and stability data.

The bioassay validation design should consider all facets of the measurement process. Sources of bioassay measurement variability include sample preparation, intra-run factors, and inter-run factors. Representative estimation of bioassay variability necessitates consideration of these factors. Test sample and Standard preparation should be performed independently during each validation run.

The replication strategy used in the validation should reflect knowledge of the factors that might influence the measurement of potency. Intra-run variability may be affected by bioassay operating factors that are usually set during development (temperature, pH, incubation times, etc.); by the bioassay design (number of animals, number of dilutions, replicates per dilution, dilution spacing, etc.); by the assay acceptance and sample acceptance criteria; and by the statistical analysis (where the primary endpoints are the similarity assessment for each sample and potency estimates for the reference samples). Operating restrictions and bioassay design (intra- and inter-run formulae that result in a reportable value for a test material) are usually specified during development and may become a part of the bioassay operating procedure. IP is studied by independent runs of the procedure, perhaps using an experimental design that alters those factors that may have an impact on the performance of the procedure. Experiments (including those that implement formalized design of experiments [DOE]) with nested or crossed design structure can reveal important sources of variability in the procedure, as well as ensure a representative estimate of long-term variability. During the validation it is not necessary to employ the format required to achieve the reportable value for a Test sample. A well-designed validation experiment that combines both intra-run and inter-run sources of variability provides estimates of independent components of the bioassay variability. These components can be used to verify or forecast the variability of the bioassay format.

A thorough analysis of the validation data should include graphical and statistical summaries of the validation parameters’ results and their conformance to target acceptance criteria. The analysis should follow the specifics of the data-analysis plan.
outlined in the validation protocol. In most cases, log relative potency should be analyzed in order to satisfy the assumptions of the statistical methods (see section 2.7 Statistical Considerations, Scale of Analysis). Those assumptions include normality of the distribution from which the data were sampled and homogeneity of variability across the range of results observed in the validation. These assumptions can be explored using graphical techniques such as box plots and probability plots. The assumption of normality can be investigated using statistical tests of normality across a suitably sized collection of historical results. Alternative methods of analysis should be sought when the assumptions can be challenged. Confidence intervals should be calculated for the validation parameters, using methods described here and in general chapter Analytical Data—Interpretation and Treatment (1010).

2.4 Validation Strategies for Bioassay Performance Characteristics

Parameters that should be verified in a bioassay are relative accuracy, specificity, IP (which incorporates repeatability), and range. Other parameters discussed in general chapter (1225) and ICH Q2(R1) such as detection limit and quantitation limit have not been included because they are usually not relevant to a bioassay that reports relative potency. These may be relevant, however, to the validation of an ancillary assay such as one used to score responders or measure response in conjunction with an in vivo potency assay. Likewise linearity is not part of bioassay validation, except as it relates to relative accuracy (dilutional linearity). There follow strategies for addressing bioassay validation parameters.

RELATIVE ACCURACY

The relative accuracy of a relative potency bioassay is the relationship between measured relative potency and known relative potency. Relative accuracy in bioassay refers to a unit slope (slope = 1) between log measured relative potency and log known relative potency. The most common approach to demonstrating relative accuracy for relative potency bioassays is by construction of target potencies by dilution of the standard material or a Test sample with known potency. This type of study is often referred to as a dilutional linearity study. The results from a dilutional linearity study should be assessed using the estimated relative bias at individual levels and via a trend in relative bias across levels. The relative bias at individual levels is calculated as follows:

\[
\text{Relative Bias} = 100 \times \frac{\text{Measured Potency}}{\text{Target Potency}} - 1 \%
\]

The trend in bias is measured by the estimated slope of log measured potency versus log target potency, which should be held to a target acceptance criterion. If there is no trend in relative bias across levels, the estimated relative bias at each level can be held to a prespecified target acceptance criterion that has been defined in the validation protocol (see section 3 A Bioassay Validation Example).

Specificity—For products or intermediates associated with complex matrices, specificity involves demonstrating lack of interference from matrix components or product-related components that can be expected to be present. This can be assessed via parallel dilution of the Standard with and without a spike addition of the potentially interfering compound. If the curves are similar and the potency conforms to expectations of a Standard-to-Standard comparison, the bioassay is specific against the compound. For these assessments both similarity and potency may be assessed using appropriate equivalence tests. Specificity may also refer to the capacity of the bioassay to distinguish between different but related biopharmaceutical molecules. An understanding should be sought of the molecule and any related forms, and of opportunities for related molecules to be introduced into the bioassay.

Intermediate Precision—Because of potential influences on the bioassay by factors such as analysts, instruments, or reagent lots, the design of the bioassay validation should include evaluation of these factors. The overall variability from measurements taken under a variety of normal test conditions within one laboratory defines the IP of the bioassay. IP is the ICH and USP term for what is also commonly referred to as inter-run variability. IP measures the influence of factors that will vary over time after the bioassay is implemented. These influences are generally unavoidable and include factors like change in personnel (new analysts), receipt of new reagent lots, etc.

When the validation has been planned using multifactor DOE, the impact of each factor can first be explored graphically to establish important contributions to potency variability. The identification of important factors should lead to procedures that seek to control their effects, such as further restrictions on intra-assay operating conditions or strategic qualification procedures on inter-run factors such as analysts, instruments, and reagent lots.

Contributions of validation study factors to the overall IP of the bioassay can be determined by performing a variance component analysis on the validation results. Variance component analysis is best carried out using a statistical software package that is capable of performing a mixed-model analysis with restricted maximum likelihood estimation (REML).

A variance component analysis yields variance component estimates such as

\[
\hat{\sigma}_{\text{intra}}^2
\]

and

\[
\hat{\sigma}_{\text{inter}}^2
\]

corresponding to intra-run and inter-run variation. These can be used to estimate the IP of the bioassay, as well as the variability of the reportable value for different bioassay formats (format variability). IP expressed as percent geometric coefficient of variation (%GCV) is given by the following formula, in this case using the natural log of the relative potency in the analysis (see section 2.7 Statistical Considerations, Scale of Analysis).
Intermediate Precision = \( 100 \cdot \left( e^{\sqrt{\frac{\sigma^2_{\text{Product}} + \sigma^2_{\text{RA}}}} k \cdot \frac{\sigma^2_{\text{RA}}}{\sigma^2_{\text{Product}}} - 1} \right) \)%

The variability of the reportable value from testing performed with \( n \) replicate sets in each of \( k \) runs (format variability) is equal to:

\[ \text{Format Variability} = 100 \cdot \left( e^{\sqrt{k \cdot \frac{\sigma^2_{\text{RA}}}{\sigma^2_{\text{Product}}} - 1}} \right) \%
\]

This formula can be used to determine a testing format suitable for various uses of the bioassay (e.g., release testing and stability evaluation).

**Range**—The range of the bioassay is defined as the true or known potencies for which it has been demonstrated that the analytical procedure has a suitable level of relative accuracy and IP. The range is normally derived from the dilutional linearity study and minimally should cover the product specification range for potency. For stability testing and to minimize having to dilute or concentrate hyper- or hypo-potent Test samples into the bioassay range, there is value in validating the bioassay over a broader range.

### 2.5 Validation Target Acceptance Criteria

The validation target acceptance criteria should be chosen to minimize the risks inherent in making decisions from bioassay measurements and to be reasonable in terms of the capability of the art. When there is an existing product specification, acceptance criteria can be justified on the basis of the risk that measurements may fall outside of the product specification. Considerations from a process capability (Cp) index can be used to inform bounds on the relative bias (RB) and the IP of the bioassay. This chapter uses the following Cpm index:

\[ \text{Cpm} = \frac{\text{USL} - \text{LSL}}{6 \cdot \sqrt{\sigma^2_{\text{Product}} + \text{RB}^2 + \sigma^2_{\text{RA}}}} \]

where USL and LSL are the upper and lower release specification, RB is a bound on the degree of relative bias in the bioassay, and

\[ \sigma^2_{\text{Product}} \]

and

\[ \sigma^2_{\text{RA}} \]

are target product variance (i.e., lot-to-lot variability) and release assay variance (with associated format) respectively. (See section 3 A Bioassay Validation Example for an example of determination of \( \sigma^2_{\text{RA}} \) and Cpm.) This formulation requires prior knowledge regarding target product variability, or the inclusion of a random selection of lots to estimate this characteristic as part of the validation. Given limited understanding of assay performance, manufacturing history, and final specifications during development, this approach may be used simply as a guide for defining validation acceptance criteria.

The choice of a bound on Cpm is a business decision. The proportion of lots that are predicted to be outside their specification limits is a function of Cpm. Some laboratories require process capability corresponding to Cpm greater than or equal to 1.3. This corresponds to approximately a 1 in 10,000 chance that a lot with potency at the center of the specification range will be outside the specification limits.

When specifications have yet to be established for a product, a restriction on relative bias or IP can be formulated on the basis of the capability of the art of the bioassay methodology. For example, although chemical assays and immunoassays are often capable of achieving near single digit percent coefficient of variation (%CV, or percent relative standard deviation, %RSD), a more liberal restriction might be placed on bioassays, such as animal potency bioassays, that operate with much larger variability (measured as %GCV which can be compared to %CV; see Appendix 1). In this case the validation goal might be to characterize the method, using the validation results to establish an assay format that is predicted to yield reliable product measurements. A sound justification for target acceptance criteria or use of characterization should be included in the validation protocol.

### 2.6 Assay Maintenance

Once a bioassay has been validated it can be implemented. However, it is important to monitor its behavior over time. This is most easily accomplished by maintaining statistical process control (SPC) charts for suitable parameters of the Standard response curve and potency of assay QC samples. The purpose of these charts is to identify at an early stage any shift or drift in the bioassay. If a trend is observed in any SPC chart, the reason for the trend should be identified. If the resolution requires a modification to the bioassay or if a serious modification of the bioassay has occurred for other reasons (for example, a major...
technology change), the modified bioassay should be revalidated or linked to the original bioassay by an adequately designed bridging study with acceptance criteria that use equivalence testing.

### 2.7 Statistical Considerations

Several statistical considerations are associated with designing a bioassay validation and analyzing the data. These relate to the properties of bioassay measurements as well as the statistical tools that can be used to summarize and interpret bioassay validation results.

#### SCALE OF ANALYSIS

The scale of analysis of bioassay validation, where data are the relative potencies of samples in the validation study, must be considered in order to obtain reliable conclusions from the study. This chapter assumes that appropriate methods are already in place to reduce the raw bioassay response data to relative potency (as described in general chapter 1034). Relative potency measurements are typically nearly log normally distributed. Log normally distributed measurements are skewed and are characterized by heterogeneity of variability, where the standard deviation is proportional to the level of response. The statistical methods outlined in this chapter require that the data be symmetric, approximating a normal distribution, but some of the procedures require homogeneity of variability in measurements across the potency range. Typically, analysis of potency after log transformation generates data that more closely fulfill both of these requirements. The base of the log transformation does not matter as long as a consistent base is maintained throughout the analysis. Thus, for example, if the natural log (log to the base e) is used to transform relative potency measurements, summary results are converted back to the bioassay scale utilizing base e.

The distribution of potency measurements should be assessed as part of bioassay development (as described in 1032). If it is determined that potency measurements are normally distributed, the validation can be carried out using methods described in the general chapter Validation of Compendial Procedures (1225).

As a consequence of the usual (for parallel-line assays) log transformation of relative potency measurements, there are advantages if the levels selected for the validation study are evenly spaced on the log scale. An example with five levels would be 0.50, 0.71, 1.00, 1.41, and 2.00. Intermediate levels are obtained as the geometric mean of two adjacent levels. For example, the mid-level between 0.50 and 1.0 is derived as follows:

\[
\text{GM} = \sqrt{0.50 \times 1.0} = 0.71
\]

Likewise, summary measures of the validation are influenced by the log normal scale. Predicted response should be reported as the geometric mean of individual relative potency measurements, and variability expressed as %GCV. GCV is calculated as the anti-log of the standard deviation, \( S_{\text{log}} \), of log transformed relative potency measurements. The formula is given by:

\[
\text{GCV} = \text{antilog}(S_{\text{log}}) - 1
\]

Variability is expressed as GCV rather than RSD of the log normal distribution in order to preserve continuity using the log transformation (see additional discussion in the Appendix 1 to this chapter). Intervals that might be calculated from GCV will be consistent with intervals calculated from mean and standard deviation of log transformed data. Table 1 presents an example of the calculation of geometric mean (GM) and associated RB, with %GCV for a series of relative potency measurements performed on samples tested at the 1.00 level. The log base e is used in the illustration.

#### Table 1. Illustration of calculations of GM and %GCV

<table>
<thead>
<tr>
<th>RP(^1)</th>
<th>In RP</th>
<th>GM</th>
<th>%GCV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1299</td>
<td>0.1221</td>
<td>1.0497</td>
<td>7.4%</td>
</tr>
<tr>
<td>0.9261</td>
<td>−0.0768</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.1299</td>
<td>0.1221</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0143</td>
<td>0.0142</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0027</td>
<td>0.0027</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0316</td>
<td>0.0311</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.1321</td>
<td>0.1241</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0499</td>
<td>0.0487</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>0.0485</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^1\) Relative potency (RP) is the geometric mean of duplicate potencies measured in the eight runs of the example given in Table 4.

Here the GM of the relative potency measurements is calculated as the anti-log of the average log relative potency measurements and then expressed as relative bias, the percent deviation from the target potency:
GM = \(e^{\text{Average}} = e^{0.0485} = 1.0497\)

\[\text{RB} = 100 \left( \frac{\text{GM}}{\text{Target}} - 1 \right) \% = 100 \left( \frac{1.0497}{1.00} - 1 \right) \% = 4.97\%
\]

and the percent geometric coefficient of variation (%GCV) is calculated as:

\[\%\text{GCV} = 100 \cdot (e^{\text{SD}} - 1) \% = 100 \cdot (e^{0.0715} - 1) \% = 7.4\%
\]

Note that the %GCV calculated for this illustration is not equal to the IP determined in the bioassay validation example for the 1.00 level (8.5%); see Table 6. This illustration utilizes the average of within-run replicates, while the IP in the validation example represents the variability of individual replicates.

**Reporting Validation Results Using Confidence Intervals**—Estimates of bioassay validation parameters should be presented as a point estimate together with a confidence interval. A **point estimate** is the numerical value obtained for the parameter, such as the GM or %GCV. A **confidence interval**’s most common interpretation is as the likely range of the true value of the parameter. The previous example determines a 90% confidence interval for average log relative potency, \(\text{Cl}_{\ln}\), as follows:

\[\text{Cl}_{\ln} = \text{Average} \pm t_{df} \cdot \frac{\text{SD}}{\sqrt{n}} - 0.0485 \pm 1.89 \cdot 0.0715/\sqrt{8} - (0.0007, 0.0963)
\]

For percent relative bias this is:

\[\text{Cl}_{\%}\text{RB} = 100 \cdot (e^{0.0007} - 1) \% = 100 \cdot (e^{0.0006} - 1) \% = (0.07\%, 10.1\%)
\]

The statistical constant (1.89) is from a t-table, with degrees of freedom (df) equal to the number of measurements minus one (df = 8 − 1 = 7). A confidence interval for IP or format variability can be formulated using methods for variance components; these methods are not covered in this general chapter.

**Assessing Conformance to Acceptance Criteria**—Bioassay validation results are compared to target acceptance criteria in order to demonstrate that the bioassay is fit for use. The process of establishing conformance of validation parameters to validation acceptance criteria should not be confused with establishing conformance of relative potency measurements to product specifications. Product specifications should inform the process of setting validation acceptance criteria.

A common practice is to apply acceptance criteria to the estimated validation parameter. This does not account, however, for the uncertainty in the estimated validation parameter. A solution is to hold the confidence interval on the validation parameter to the acceptance criterion. This is a standard statistical approach used to demonstrate conformance to expectation and is called an **equivalence test**. It should not be confused with the practice of performing a significance test, such as a t-test, which seeks to establish a difference from some target value (e.g., 0% relative bias). A significance test associated with a P-value > 0.05 (equivalent to a confidence interval that includes the target value for the parameter) indicates that there is insufficient evidence to conclude that the parameter is different from the target value. This is not the same as concluding that the parameter conforms to its target value. The study design may have too few replicates, or the validation data may be too variable to discover a meaningful difference from target. Additionally, a significance test may detect a small deviation from target that is of negligible importance. These scenarios are illustrated in Figure 1.

![Figure 1](image-url)

**Figure 1.** Use of confidence intervals to establish that validation results conform to an acceptance criterion.

The solid horizontal line represents the target value (perhaps 0% relative bias), and the dashed lines form the lower (LAL) and upper (UAL) acceptance limits. In scenario a, the confidence bound includes the target, and thus one could conclude there is insufficient evidence to conclude a difference from target (the significance test approach). However, although the point estimate (the solid diamond) falls within the acceptance range, the interval extends outside the range, which signifies that the true relative bias may be outside the acceptable range. In scenario b, the interval falls within the acceptance range, signifying conformance to the acceptance criterion. The interval in scenario c also falls within the acceptance range but excludes the
target. Thus, for scenario c, although the difference of the point estimate from the target is statistically significant, c is acceptable because the confidence interval falls within the target acceptance limits.

Using the 90% confidence interval calculated previously, we can establish whether the bioassay has acceptable relative bias at the 1.00 level compared to a target acceptance criterion of no greater than +12%, for example. Because the 90% confidence interval for percent relative bias (0.07%, 10.1%) falls within the interval (100*[1/1.12 −1]%, 100*[1.12/1−1]%) = (−11%, 12%), we conclude that there is acceptable relative bias at the 1.00 level. Note that a 90% confidence interval is used in an equivalence test rather than a conventional 95% confidence interval. This is common practice and is the same as the two one-sided tests (TOST) approach used in pharmaceutical bioequivalence testing.

**Risks in Decision-Making and Number of Validation Runs**—The application of statistical tests, including the assessment of conformance of a validation parameter to its acceptance criteria, involves risks. One risk is that the parameter does not meet its acceptance criterion although the property associated with that parameter is satisfactory; another, the converse, is that the parameter meets its acceptance criterion although the parameter is truly unsatisfactory. A consideration related to these risks is sample size.

The two types of risk can be simultaneously controlled via strategic design, including choice of the number of runs that will be conducted in the validation. Specifically, the minimum number of runs needed to establish conformance to an acceptance criterion for relative bias is given by:

\[
n \geq \frac{(t_{\alpha, df} + t_{\beta/2, df})^2 \sigma_{IP}^2}{\theta^2}
\]

where \(t_{\alpha, df}\) and \(t_{\beta/2, df}\) are distributional points from a Student’s t-distribution; \(\alpha\) and \(\beta\) are the one-sided type I and type II errors, and represent the risks associated with drawing the wrong conclusion in the validation; \(df\) is the degrees of freedom associated with the study design (usually \(n - 1\));

\[
\sigma_{IP}^2
\]

is a preliminary estimate of IP; and \(\theta\) is the acceptable deviation (target acceptance criterion).

For example, if the acceptance criterion for relative bias is ±0.11 log (i.e., \(\theta = 0.11\)), the bioassay variability is

\[
\hat{\sigma}_{IP} = 0.076 \text{ log}
\]

and \(\alpha = \beta = 0.05\),

\[
n \geq \frac{(1.89 + 2.36)^2 \cdot 0.076^2}{0.11^2} = 8 \text{ runs}
\]

Note that this formulation of sample size assumes no intrinsic bias in the bioassay. A more conservative solution includes some nonzero bias in the determination of a sample size. This results in a greater sample size to offset the impact of the bias on the conclusions of the validation. In the current example the sample size increases to 10 runs if one assumes an intrinsic bias equal to 2%. Note also that this calculation represents a recursive solution (because the degrees of freedom depend on \(n\)) requiring statistical software or an algorithm that employs iterative methodology.

Note further that the selection of \(\alpha\) and \(\beta\) should be justified on the basis of the corresponding risks of drawing the wrong conclusion from the validation.

**Modeling Validation Results Using Mixed Effects Models**—Many analyses associated with bioassay validation must account for multiple design factors such as fixed effects (e.g., potency level), as well as random effects (e.g., analyst, run, and replicate). Statistical models composed of both fixed and random effects are called mixed effects models and usually require sophisticated statistical software for analysis. The results of the analysis may be summarized in an analysis of variance (ANOVA) table or a table of variance component estimates. The primary goal of the analysis is to estimate critical parameters rather than establish the significance of an effect. The modeling output provides parameter estimates together with their standard errors of estimates that can be utilized to establish conformance of a validation parameter to its acceptance criterion. Thus the average relative bias at each level is obtained as a portion of the analysis together with its associated variability. These compose a confidence interval that is compared to the acceptance criterion as described above. If variances across levels can be pooled, statistical modeling can also determine the overall relative bias and IP by combining information across levels performed in the validation. Similarly, mixed effects models can be used to obtain variance components for validation study factors and to combine results across validation study samples and levels.

**Statistical Design**—Statistical designs, such as multifactor DOE or nesting, can be used to organize assay and runs in a bioassay validation. It is useful to incorporate factors that are believed to influence the bioassay response and that vary during long-term use of the procedure into these designs. Using these methods of design, the sources of variability may be characterized and a strategic test plan to manage the variability of the bioassay may be developed.

Table 2 shows an example of a multifactor DOE that incorporates multiple analysts, multiple cell culture preparations, and multiple reagent lots into the validation plan.

<table>
<thead>
<tr>
<th>Run</th>
<th>Analyst</th>
<th>Cell Prep</th>
<th>Reagent Lot</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Published on March 26, 2020
Table 2. Example of a Multifactor DOE with 3 Factors (continued)

<table>
<thead>
<tr>
<th>Run</th>
<th>Analyst</th>
<th>Cell Prep</th>
<th>Reagent Lot</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>7</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>8</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

In this design each analyst performs the bioassay with both cell preparations and both reagent lots. This is an example of a full factorial design because all combinations of the factors are performed in the validation study. To reduce the number of runs in the study, fractional factorial designs may be employed when more than three factors have been identified. For example, if it is practical for an analyst to perform four assays in a run, a split-unit design could be used with analysts as the whole-plot factor and cell preparation and reagent lot as sub-plot factors. Unlike screening experiments, the validation design should incorporate as many factors at as many levels as possible in order to obtain a representative estimate of IP. More than two levels of a factor should be employed in the design whenever possible. This may be accomplished in a less structured manner, without regard to strict factorial layout. Validation runs should be randomized whenever possible to mitigate the potential influences of run order or time.

Figure 2 illustrates an example of a validation using nesting (replicates nested within plate, plate nested within analyst).

For both of these types of design as well as combinations of the two, components of variability can be estimated from the validation results. These components of variability can be used to identify significant sources of variability as well as to derive a bioassay format that meets the procedure’s requirements for precision. It should be noted that significant sources of variability may have been identified during bioassay development. In this case the validation should confirm both the impact of these factors and the assay format that meets the requirement for precision.

Significant Figures—The number of significant figures in a reported result from a bioassay is related to the latter’s precision. In general, a bioassay with %GCV between 2% and 20% will support two significant figures. The number of significant figures should not be confused with the number of decimal places—reported values equal to 1.2 and 0.12 have the same number (two) of significant figures. This standard of rounding is appropriate for log scaled measurements that have constant variation on the log scale and proportional rather than additive variability on the original scale (or the scale commonly used for interpretation). Note that rounding occurs at the end of a series of calculations when the final measurement is reported and used for decision making such as conformance to specifications. Thus if the final measurement is a reportable value from multiple assays, rounding should not occur prior to determination of the reportable value. Likewise, specifications should be stated with the appropriate number of significant figures.

3. A BIOASSAY VALIDATION EXAMPLE

An example illustrates the principles described in this chapter. The bioassay will be used to support a specification range of 0.71 to 1.41 for the product. Using the Cpm described in section 2.5 Validation Target Acceptance Criteria, a table is derived showing the projected rate of OOS results for various restrictions on RB and IP. Cpm is calculated on the basis of the variability of a reportable value using three independent runs of the bioassay (see discussion of format variability, above). Product variability is assumed to be equal to 0 in the calculations. The laboratory may wish to include target product variability. An estimate of target product variability can be obtained from data from a product, for example, manufactured by a similar process.

<table>
<thead>
<tr>
<th>LSL-USL</th>
<th>IP (%)</th>
<th>RB (%)</th>
<th>Cpm</th>
<th>Prob(OOS) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.71–1.41</td>
<td>20</td>
<td>20</td>
<td>0.54</td>
<td>10.5</td>
</tr>
<tr>
<td>0.71–1.41</td>
<td>8</td>
<td>12</td>
<td>0.94</td>
<td>0.48</td>
</tr>
<tr>
<td>0.71–1.41</td>
<td>10</td>
<td>5</td>
<td>1.55</td>
<td>0.0003</td>
</tr>
</tbody>
</table>

The calculation is illustrated for IP equal to 8% and relative bias equal to 12% (n = 3 runs):
\[
C_{pm} = \frac{\ln(1.41) - \ln(0.71)}{6 \cdot \sqrt{[\ln(1.08)]^2 / 3 + [\ln(1.12)]^2}} = 0.94
\]

\[
\text{Prob}(OOS) = 2 \cdot \Phi(-3 \cdot 0.94) = 0.0048 \ (0.48\%),
\]

where \( \Phi \) represents the standard normal cumulative distribution function.

From Table 3, acceptable performance (less than 1\% chance of obtaining an OOS result due to bias and variability of the bioassay) can be expected if the IP is \( \leq 8\% \) and relative bias is \( \leq 12\% \). The sample size formula given in section 2.7 Statistical Considerations, Risks in Decision-Making and Number of Validation Runs can be used to derive the number of runs required to establish conformance to an acceptance criterion for relative bias equal to 12\% (using \( \%GCV_p = 8\%; \ \alpha = \beta = 0.05 \)):

\[
n \geq \frac{(1.89 + 2.36)^2 \cdot [\ln(1.08)]^2}{[\ln(1.12)]^2} \approx 8 \text{ runs}
\]

Thus eight runs would be needed in order to have a 95\% chance of passing the target acceptance criterion for relative bias if the true relative bias is zero. Note that the calculation of sample size assumes that a singlet of the validation samples will be performed in each validation run. The use of multiple replication sets and/or multiple assays will provide valuable information that allows separate estimates for intra-run and inter-run variability, and will decrease the risk of failing to meet the validation target acceptance criteria.

Five levels of the target analyte are studied in the validation: 0.50, 0.71, 1.00, 1.41, and 2.00. Two runs at each level are generated by two trained analysts using two media lots. Other factors may be considered and incorporated into the design using a fractional factorial layout. The laboratory should strive to design the validation with as many levels of each factor as possible in order to best model the long-term performance of the bioassay. In this example each analyst performs two runs at each level using each media lot. A run consists of a full dilution series of the Standard as described in the bioassay’s operating procedure, together with two independent dilution series of the Test sample. This yields duplicate measurements of relative potency in each run; see Table 4 for all relative potency observations. Note that the two potency estimates at each level of potency in a run are not independent due to common analysts and media lots.

### Table 4. Example of Bioassay Validation with Two Analysts, Two Media Lots, and Runs per Level for Each Combination of Analyst and Lot

<table>
<thead>
<tr>
<th>Media Lot/Analyst</th>
<th>1/1</th>
<th>1/2</th>
<th>2/1</th>
<th>2/2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Run</td>
<td>1/1</td>
<td>1/2</td>
<td>2/1</td>
<td>2/2</td>
</tr>
<tr>
<td>0.50</td>
<td>0.5215</td>
<td>0.4532</td>
<td>0.5667</td>
<td>0.5054</td>
</tr>
<tr>
<td>0.71</td>
<td>0.7558</td>
<td>0.6689</td>
<td>0.6843</td>
<td>0.7050</td>
</tr>
<tr>
<td>1.00</td>
<td>0.7082</td>
<td>0.6182</td>
<td>0.8217</td>
<td>0.7143</td>
</tr>
<tr>
<td>1.41</td>
<td>1.1052</td>
<td>0.9774</td>
<td>1.1527</td>
<td>0.9901</td>
</tr>
<tr>
<td>2.00</td>
<td>2.3529</td>
<td>1.8883</td>
<td>2.3501</td>
<td>2.2906</td>
</tr>
<tr>
<td>2.00</td>
<td>2.2307</td>
<td>1.9813</td>
<td>2.4013</td>
<td>2.1725</td>
</tr>
</tbody>
</table>

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A plot is used to reveal irregularities in the experimental results. In particular, a properly prepared plot can reveal a failure in agreement of validation results with validation levels, as well as heterogeneity of variability across levels (see discussion of the log transformation in section 2.7 Statistical Considerations). The example plot in Figure 3 includes the unit line (line with slope equal to 1, passing through the origin). The analyst 1 and analyst 2 data are deliberately offset with respect to the expected potency to allow clear visualization and comparison of the data sets from each analyst.

A formal analysis of the validation data might be undertaken in the following steps: (1) an assessment of variability (IP) should precede an assessment of relative accuracy or specificity in order to establish conformance to the assumption that variances across sample levels can be pooled; and (2) relative accuracy is assessed either at separate levels or by a combined analysis, depending on how well the data across levels can be pooled. These steps are demonstrated using the example validation data, along with some details of the calculations for illustrative purposes. Note that the calculations illustrated in the following sections are appropriate only with a balanced dataset. Imbalanced designs or datasets with missing relative potency measurements should be analyzed using a mixed model analysis with restricted maximum likelihood estimation (REML).

### 3.1 Intermediate Precision

Data at each level can be analyzed using variance component analysis. With balanced data, as in this example, variance components can be determined from a standard one-way ANOVA. An example of the calculation performed at a single level (0.50) is presented in Table 5.

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>Expected Mean Square</th>
</tr>
</thead>
<tbody>
<tr>
<td>Run</td>
<td>7</td>
<td>0.05317</td>
<td>0.007902</td>
<td>Var(Error) + 2 Var(Run)</td>
</tr>
<tr>
<td>Error</td>
<td>8</td>
<td>0.006130</td>
<td>0.000766</td>
<td>Var(Error)</td>
</tr>
<tr>
<td>Corrected total</td>
<td>15</td>
<td>0.061447</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The top of the table represents a standard ANOVA analysis. Analyst and media lot have not been included because of the small number of levels (2 levels) for each factor. The factor “Run” in this analysis represents the combined runs across the analyst by media lot combinations. The Expected Mean Square is the linear combination of variance components that generates the measured mean square for each source. The variance component estimates are derived by solving the equation “Expected Mean Square = Mean Square” for each component. To start, the mean square for Error estimates Var(Error), the within-run component of variability, is

\[
\text{Var(Error)} = \text{MSE(Error)} = 0.000766
\]

The between-run component of variability, Var(Run), is subsequently calculated by setting the mean square for Run to the mathematical expression for the expected mean square, then solving the equation for Var(Run) as follows:
These variance component estimates are combined to establish the overall IP of the bioassay at 0.50:

\[
IP = 100 \cdot \left( e^{\frac{\text{Var(Run)} + \text{Var(Error)}}{2}} - 1 \right)\%
\]

\[
= 100 \cdot \left( e^{\frac{0.003568 + 0.000648}{2}} - 1 \right)\% = 6.8\%
\]

The same analysis was performed at each level of the validation, and is presented in Table 6.

### Table 6. Variance Component Estimates and Overall Variability for Each Validation Level and the Average

<table>
<thead>
<tr>
<th>Component</th>
<th>0.50</th>
<th>0.71</th>
<th>1.00</th>
<th>1.41</th>
<th>2.00</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Var(Run)</td>
<td>0.003568</td>
<td>0.000648</td>
<td>0.003639</td>
<td>0.003135</td>
<td>0.002623</td>
<td>0.002723</td>
</tr>
<tr>
<td>Var(Error)</td>
<td>0.000766</td>
<td>0.004303</td>
<td>0.002954</td>
<td>0.000577</td>
<td>0.002258</td>
<td>0.002172</td>
</tr>
<tr>
<td>Overall</td>
<td>6.8%</td>
<td>7.3%</td>
<td>8.5%</td>
<td>6.3%</td>
<td>7.2%</td>
<td>7.2%</td>
</tr>
</tbody>
</table>

A combined analysis can be performed if the variance components are similar across levels. Typically a heuristic method is used for this assessment. One might hold the ratio of the maximum variance to the minimum variance to no greater than 10 (10 is used because of the limited number of runs performed in the validation). Here the ratios associated with the between-run variance component, 0.003639/0.000648 = 5.6, and the within-run component, 0.004303/0.000577 = 7.5, meet the 10-fold criterion. Had the ratio exceeded 10 and if this was due to excess variability in one or the other of the extremes in the levels tested, that extreme would be eliminated from further analysis and the range would be limited to exclude that level.

The analysis might proceed using statistical software that is capable of applying a mixed effects model to the validation results. That analysis should account for any imbalance in the design, random effects such as analyst and media lot, and fixed effects such as level (see section 2.7 Statistical Considerations, Modeling Validation Results Using Mixed Effects Models). Variance components can be determined for analyst and media lot separately in order to characterize their contributions to the overall variability of the bioassay.

In the example, variance components can be averaged across levels to report the IP of the bioassay. This method of combining estimates is exact only if a balanced design has been employed in the validation (i.e., the same replication strategy at each level). A balanced design was employed for the example validation, so the IP can be reported as 7.2% GCV.

Because of the recommendation to report validation results with some measure of uncertainty, a one-sided 95% upper confidence bound can be calculated for the IP of the bioassay. The literature contains methods for calculating confidence bounds for variance components. The upper bound on IP for the bioassay example is 11.8% GCV. The upper confidence bound was not calculated at each level separately because of the limited data at an individual level relative to the overall study design.

### 3.2 Relative Accuracy

The analysis might proceed with an assessment of relative accuracy at each level. Table 7 shows the average and 90% confidence interval of validation results in the log scale, as well as corresponding potency and relative bias.

### Table 7. Average Potency and Relative Bias at Individual Levels

<table>
<thead>
<tr>
<th>Level</th>
<th>n *</th>
<th>Log Potency</th>
<th>(90% CI)</th>
<th>Potency</th>
<th>(90% CI)</th>
<th>Average Potency</th>
<th>(90% CI)</th>
<th>Relative Bias</th>
<th>(90% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.50</td>
<td>8</td>
<td>-0.6613</td>
<td>(-0.7034, -0.6192)</td>
<td>0.52</td>
<td>(0.49, 0.54)</td>
<td>3.23%</td>
<td>(-1.02, 7.67)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.71</td>
<td>8</td>
<td>-0.3419</td>
<td>(-0.3773, -0.3064)</td>
<td>0.71</td>
<td>(0.69, 0.74)</td>
<td>0.06%</td>
<td>(-3.42, 3.67)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.00*</td>
<td>8</td>
<td>0.0485</td>
<td>(0.0006, 0.0964)</td>
<td>1.05</td>
<td>(1.00, 1.10)</td>
<td>4.97%</td>
<td>(0.06, 10.12)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.41</td>
<td>8</td>
<td>0.3723</td>
<td>(0.3331, 0.4115)</td>
<td>1.45</td>
<td>(1.40, 1.51)</td>
<td>2.91%</td>
<td>(-1.04, 7.03)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.00</td>
<td>8</td>
<td>0.7859</td>
<td>(0.7449, 0.8269)</td>
<td>2.19</td>
<td>(2.11, 2.29)</td>
<td>9.72%</td>
<td>(5.31, 14.32)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Analysis performed on averages of duplicates from each run.

* Calculation illustrated in section 2.7 Statistical Considerations, Scale of Analysis.

The analysis has been performed on the average of the duplicates from each run (n = 8 runs) because duplicate measurements are correlated within a run by shared IP factors (analyst, media lot, and run in this case). A plot of relative bias versus level can be used to examine patterns in the experimental results and to establish conformance to the target acceptance criterion for relative bias (12%).
Figure 4 shows an average positive bias across sample levels (i.e., the average relative bias is positive at all levels). This consistency is due in part to the lack of independence of bioassay results across levels. In addition there does not appear to be a trend in relative bias across levels. The latter would indicate that a comparison of samples with different measured relative potency (such as stability samples) is biased, resulting perhaps in an erroneous conclusion. Trend analysis can be performed using a regression of log relative potency versus log level. Introduction during the development of the bioassay validation protocol of an acceptance criterion on a trend in relative accuracy across the range can be considered.

After establishing that there is no meaningful trend across levels, the analysis proceeds with an assessment of the relative accuracy at each level. The bioassay has acceptable relative bias at levels from 0.50 to 1.41, yielding 90% confidence bounds (equivalent to a two one-sided t-test) that fall within the acceptance region of −11% to 12% relative bias. The 90% confidence interval at 2.0 falls outside the acceptance region, indicating that the relative bias may exceed 12%.

A combined analysis can be performed utilizing statistical software that is capable of applying a mixed effects model to the validation results. That analysis accurately accounts for the validation study design. The analysis also accommodates random effects such as analyst, media lot, and run (see section 2.7 Statistical Considerations, Modeling Validation Results Using Mixed Effects Models).

### 3.3 Range

The conclusions derived from the assessment of IP and relative accuracy can be used to establish the bioassay's range that demonstrates satisfactory performance. Based on the acceptance criterion for IP equal to 8% GCV (see Table 6) and for relative bias equal to 12% (see Table 7), the range of the bioassay is 0.50 to 1.41. In this range, level 1.0 has a slightly higher than acceptable estimate of IP (8.5% versus the target acceptance criterion ≤ 8.0%), which may be due to the variability of the estimate that results from a small dataset. Because of this and other results in Table 6, one may conclude that satisfactory IP was demonstrated across the range.

### 3.4 Use of Validation Results for Bioassay Characterization

When the study has been performed to estimate the characteristics of the bioassay (characterization), the variance component estimates can also be used to predict the variability for different bioassay formats and thereby can determine a format that has a desired level of precision. The predicted variability for k independent runs, with n individual dilution series of the test preparation within a run, is given by the following formula for format variability:

\[
\text{Format Variability} = 100 \cdot (e^{\sqrt{\text{Var}(\text{Run})/k + \text{Var}(\text{Error})/(nk)}} - 1)
\]

Using estimates of intra-run and inter-run variance components from Table 6 [Var(Run) = 0.002723 and Var(Error) = 0.002172], if the bioassay is performed in three independent runs, the predicted variability of the reportable value (geometric mean of the relative potency results) is equal to:

\[
\text{Format Variability} = 100 \cdot (e^{0.002723/3 + 0.002172/(1 \cdot 3)}} - 1) = 4.1\%
\]

This calculation can be expanded to include various combinations of runs and minimal sets (assuming that the numbers of samples, dilutions, and replicates in the minimal sets are held constant) within runs as shown in Table 8.

<table>
<thead>
<tr>
<th>Reps (n)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7.2%</td>
<td>5.1%</td>
<td>4.1%</td>
<td>2.9%</td>
</tr>
<tr>
<td>2</td>
<td>6.4%</td>
<td>4.5%</td>
<td>3.6%</td>
<td>2.6%</td>
</tr>
<tr>
<td>3</td>
<td>6.0%</td>
<td>4.2%</td>
<td>3.4%</td>
<td>2.4%</td>
</tr>
<tr>
<td>6</td>
<td>5.7%</td>
<td>4.0%</td>
<td>3.3%</td>
<td>2.3%</td>
</tr>
</tbody>
</table>

*Not official text. Please refer to the currently official version of the applicable USP–NF or FCC standard for compliance purposes.*

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Clearly the most effective means of reducing the variability of the reportable value (the geometric mean potency across runs and minimal sets) is by independent runs of the bioassay procedure. In addition, confidence bounds on the variance components used to derive IP can be utilized to establish the bioassay’s format variability. Significant sources of variability must be incorporated into runs in order to effect variance reduction. A more thorough analysis of the bioassay validation example would include analyst and media lot as factors in the statistical model. Variance component estimates obtained from such an analysis are presented in Table 9.

### Table 9. REML Estimates of Variance Components Associated with Analyst, Media Lot, and Run

<table>
<thead>
<tr>
<th>Variance</th>
<th>Component Estimate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Var(Media Lot)</td>
<td>0.0000</td>
</tr>
<tr>
<td>Var(Analyst)</td>
<td>0.0014</td>
</tr>
<tr>
<td>Var(Analyst*Media Lot)</td>
<td>0.0000</td>
</tr>
<tr>
<td>Var(Run (Analyst*Media Lot))</td>
<td>0.0019</td>
</tr>
<tr>
<td>Var(Error)</td>
<td>0.0022</td>
</tr>
</tbody>
</table>

Identification of analyst as a significant bioassay factor should ideally be addressed during bioassay development. Nonetheless the laboratory may choose to address the apparent contribution of analyst-to-analyst variability through improved training or by using multiple analysts in formatting the assay for routine performance of the bioassay. Estimates of intra-run and inter-run variability can also be used to determine the sizes of differences (fold difference) that can be distinguished between samples tested in the bioassay. For k runs, with n minimal sets within each run, using an approximate two-sided critical value from the standard normal distribution with z = 2, the critical fold difference between reportable values for two samples that are tested in the same runs of the bioassay is given by:

\[
\text{Critical Fold Difference} = e^{2 \cdot \sqrt{Var(\text{Run})/k + Var(\text{Error})/(nk)}}
\]

When samples have been tested in different runs of the bioassay (such as long-term stability samples), the critical fold difference is given by (assuming the same format is used to test the two series of samples):

\[
\text{Critical Fold Difference} = e^{2 \cdot \sqrt{Var(\text{Run}) + Var(\text{Error})/(nk)}}
\]

For comparison of samples the laboratory can choose a design (bioassay format) that has suitable precision to detect a practically meaningful fold difference between samples.

### 3.5 Confirmation of Intermediate Precision and Revalidation

The estimate of IP from the validation is highly uncertain because of the small number of runs performed. After the laboratory gains suitable experience with the bioassay, the estimate can be confirmed or updated by analysis of control sample measurements such as the variability of a positive control. This analysis can be done with the control prepared and tested like a Test sample (i.e., same or similar dilution series and replication strategy). This assessment should be made after sufficient assays have been performed to obtain an alternative estimate of the bioassay’s intermediate precision, including implementation of changes (e.g., different analysts, different key reagent lots, and different cell preparations) associated with the standardized assay protocol. The reported IP of the bioassay should be modified as an amendment to the validation report if the assessment reveals a substantial disparity of results.

The bioassay should be revalidated whenever a substantial change is made to the method. This includes but is not limited to a change in technology or a change in readout. The revalidation may consist of a complete re-enactment of the bioassay validation or a bridging study that compares the current and the modified methods.

### APPENDICES

#### Appendix 1: Measures of Location and Spread for Log Normally Distributed Variables

Two assumptions of common statistical procedures, such as ANOVA or confidence interval estimation, are (1) the variation in the bioassay response about its mean is normally distributed and (2) the standard deviation of the observed response values is constant over the range of responses that are of interest. Such responses are said to have a “normal distribution” and an “additive error structure”. When these two conditions are not met, it may be useful to consider a transformation before using common statistical procedures.

The variation in bioassay responses is often found to be non-normal (skewed toward higher values) with a standard deviation approximately proportional (or nearly so) to the mean response. Such responses often have a “multiplicative error structure” and follow a “log normal distribution” with a percent coefficient of variation (%CV) that is constant across the response range of interest. In such cases, a log transformation of the bioassay response will be found to be approximately normal with a nearly constant standard deviation over the response range. After log transformation, then, the two assumptions are met, and common statistical procedures can be performed on the log transformed response. The following discussion presumes a log normal distribution for the bioassay response.

We refer to an observed bioassay response value, X, as being on the “original scale of measurement” and to the log transformed response, Y = log(X), as being on the “log transformed scale”. Although common statistical procedures may be
appropriate only on the log transformed scale, we can summarize bioassay response results by estimating measures of location (e.g., mean or median), measures of spread (e.g., standard deviation), or confidence intervals on either scale of measurement, as long as the scale being used is indicated. The %CV is useful on the original scale where it is constant over the response range. For the same reason, the standard deviation (SD) is relevant on the log transformed scale. There may be advantages to reporting statistical summaries on the basis of the log transformed (Y) scale. However, it is often informative to back transform the reported measures to the original scale of measurement (X).

For any given value of X, there is only one unique value of Y = log(X), and vice versa. Similarly for measures of location and spread, there is a unique one-to-one correspondence between measures of location and spread obtained on the original and log transformed scales. Further, just as there is a simple relationship between X and Y = log(X), there are relatively simple relationships that allow conversion between the corresponding measures on each scale, as indicated in Table A-1 below. In the table, “Average” and “SD”, wherever they appear, refer to measures calculated on the log transformed (Y) scale.

<table>
<thead>
<tr>
<th>Measure</th>
<th>Scale of Measurement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Location</td>
<td>Log Transformed (V)</td>
</tr>
<tr>
<td>Mean (average)</td>
<td>Geometric mean (GM)</td>
</tr>
<tr>
<td>Spread</td>
<td>Original (X)</td>
</tr>
<tr>
<td>Standard deviation (SD)</td>
<td>Geometric standard deviation (GSD) = e^{SD}</td>
</tr>
<tr>
<td>Confidence intervals (k is an appropriate constant based on the t-distribution or large sample z approximation)</td>
<td>Lower Average – k · SD/√n</td>
</tr>
<tr>
<td></td>
<td>Upper Average + k · SD/√n</td>
</tr>
<tr>
<td></td>
<td>Size Width (upper – lower) = 2 · k · SD/√n</td>
</tr>
<tr>
<td>Percent coefficient of variation (%CV)</td>
<td>%GCV = 100 · (GSD – 1)</td>
</tr>
</tbody>
</table>

The geometric mean (GM) should not be misinterpreted as an estimate of the mean of the original scale (X) variable, but is instead an estimate of the median of X. The median is a more appropriate measure of location for variables with skewed error distributions such as the log normal, as well as symmetric error distributions where the median is equal to the mean.

Similarly, the geometric standard deviation (GSD) should not be misinterpreted as the standard deviation of the original scale (X) variable. GSD is, however, a useful multiplicative factor for obtaining confidence intervals on the original (X) scale that correspond to those on the log transformed (Y) scale, as shown in the above table. A GSD of 1 corresponds to no variation (SD of Y = 0). The ratio of the Upper to the Lower confidence bounds, on the untransformed (X) scale, will be equal to GSD^{2k/√n}, as can be seen from Table A-1.

The geometric coefficient of variation (%GCV) approximates the %CV on the original (X) scale when the %CV is below 20%. It is important not to confuse these different measures of spread. The %GCV is a measure relevant to the log transformed (Y) scale, and the %CV is a measure relevant to the original (X) scale. Depending on the preferred frame of reference, either or both measures may be useful.

**Appendix 2: Information Sources**


**Appendix 3: Additional Sources of Information**

Additional information and alternative methods can be found in the references listed below.


**1034** ANALYSIS OF BIOLOGICAL ASSAYS

1. INTRODUCTION

Although advances in chemical characterization have reduced the reliance on bioassays for many products, bioassays are still essential for the determination of potency and the assurance of activity of many proteins, vaccines, complex mixtures, and products for cell and gene therapy, as well as for their role in monitoring the stability of biological products. The intended scope of the general chapter on Analysis of Biological Assays (1034) includes guidance for the analysis of results both of bioassays described in the *United States Pharmacopeia* (USP), and of non-USP bioassays that seek to conform to the qualities of bioassay analysis recommended by USP. Note the emphasis on analysis—design and validation are addressed in complementary chapters (Development and Design of Bioassays (1032) and Biological Assay Validation (1033), respectively).

Topics addressed in (1034) include statistical concepts and methods of analysis for the calculation of potency and confidence intervals for a variety of relative potency bioassays, including those referenced in USP. Chapter (1034) is intended for use primarily by those who do not have extensive training or experience in statistics and by statisticians who are not experienced in the analysis of bioassays. Sections that are primarily conceptual require only minimal statistics background. Most of the chapter and all the methods sections require that the nonstatistician be comfortable with statistics at least at the level of USP general chapter Analytical Data—Interpretation and Treatment (1010) and with linear regression. Most of sections 3.4 Nonlinear Models for Quantitative Response and 3.6 Dichotomous (Quantal) Assays require more extensive statistics background and thus are intended primarily for statisticians. In addition, (1034) introduces selected complex methods, the implementation of which requires the guidance of an experienced statistician.

Approaches in (1034) are recommended, recognizing the possibility that alternative procedures may be employed. Additionally, the information in (1034) is presented assuming that computers and suitable software will be used for data analysis. This view does not relieve the analyst of responsibility for the consequences of choices pertaining to bioassay design and analysis.

2. OVERVIEW OF ANALYSIS OF BIOASSAY DATA

Following is a set of steps that will help guide the analysis of a bioassay. This section presumes that decisions were made following a similar set of steps during development, checked during validation, and then not required routinely. Those steps and decisions are covered in general information chapter Design and Development of Biological Assays (1032). Section 3 Analysis Models provides details for the various models considered.

1. As a part of the chosen analysis, select the subset of data to be used in the determination of the relative potency using the prespecified scheme. Exclude only data known to result from technical problems such as contaminated wells, non-monotonic concentration–response curves, etc.
2. Fit the statistical model for detection of potential outliers, as chosen during development, including any weighting and transformation. This is done first without assuming similarity of the Test and Standard curves but should include important elements of the design structure, ideally using a model that makes fewer assumptions about the functional form of the response than the model used to assess similarity.
3. Determine which potential outliers are to be removed and fit the model to be used for suitability assessment. Usually, an investigation of outlier cause takes place before outlier removal. Some assay systems can make use of a statistical (noninvestigative) outlier removal rule, but removal on this basis should be rare. One approach to “rare” is to choose the outlier rule so that the expected number of false positive outlier identifications is no more than one; e.g., use a 1% test if the sample size is about 100. If a large number of outliers are found above that expected from the rule used, that calls into question the assay.
4. Assess system suitability. System suitability assesses whether the assay Standard preparation and any controls behaved in a manner consistent with past performance of the assay. If an assay (or a run) fails system suitability, the entire assay (or run) is discarded and no results are reported other than that the assay (or run) failed. Assessment of system suitability usually includes adequacy of the fit of the model used to assess similarity. For linear models, adequacy of the model may include assessment of the linearity of the Standard curve. If the suitability criterion for linearity of the Standard is not met, the exclusion of one or more extreme concentrations may result in the criterion being met. Examples of other possible system suitability criteria include background, positive controls, max/min, max/background, slope, IC50 (or EC50), and variation around the fitted model.
5. Assess sample suitability for each Test sample. This is done to confirm that the data for each Test sample satisfy necessary assumptions. If a Test sample fails sample suitability, results for that sample are reported as “Fails Sample Suitability.” Relative potencies for other Test samples in the assay may still be reported. Most prominent of sample suitability criteria...
is similarity, whether parallelism for parallel models or equivalence of intercepts for slope-ratio models. For nonlinear models, similarity assessment involves all curve parameters other than EC_{50} (or IC_{50}).

6. For those Test samples in the assay that meet the criterion for similarity to the Standard (i.e., sufficiently similar concentration–response curves or similar straight-line subsets of concentrations), calculate relative potency estimates assuming similarity between Test and Standard, i.e., by analyzing the Test and Standard data together using a model constrained to have exactly parallel lines or curves, or equal intercepts.

7. A single assay is often not sufficient to achieve a reportable value, and potency results from multiple assays can be combined into a single potency estimate. Repeat steps 1–6 multiple times, as specified in the assay protocol or monograph, before determining a final estimate of potency and a confidence interval.

8. Construct a variance estimate and a measure of uncertainty of the potency estimate (e.g., confidence interval). See section 4 Confidence Intervals.

A step not shown concerns replacement of missing data. Most modern statistical methodology and software do not require equal numbers at each combination of concentration and sample. Thus, unless otherwise directed by a specific monograph, analysts generally do not need to replace missing values.

### 3. ANALYSIS MODELS

A number of mathematical functions can be successfully used to describe a concentration–response relationship. The first consideration in choosing a model is the form of the assay response. Is it a number, a count, or a category such as Dead/Alive? The form will identify the possible models that can be considered.

Other considerations in choosing a model include the need to incorporate design elements in the model and the possible benefits of means models compared to regression models. For purposes of presenting the essentials of the model choices, section 3 Analysis Models assumes a completely randomized design so that there are no design elements to consider and presents the models in their regression form.

#### 3.1 Quantitative and Qualitative Assay Responses

The terms quantitative and qualitative refer to the nature of the response of the assay used in constructing the concentration–response model. Assays with either quantitative or qualitative responses can be used to quantify product potency. Note that the responses of the assay at the concentrations measured are not the relative potency of the bioassay. Analysts should understand the differences among responses, concentration–response functions, and relative potency.

A quantitative response results in a number on a continuous scale. Common examples include spectrophotometric and luminescence responses, body weights and measurements, and data calculated relative to a standard curve (e.g., cytokine concentration). Models for quantitative responses can be linear or nonlinear (see sections 3.2–3.5).

A qualitative measurement results in a categorical response. For bioassay, qualitative responses are most often quantal, meaning they entail two possible categories such as Positive/Negative, 0/1, or Dead/Alive. Quantal responses may be reported as proportions (e.g., the proportion of animals in a group displaying a property). Quantal models are presented in section 3.6. Qualitative responses can have more than two possible categories, such as end-point titer assays. Models for more than two categories are not considered in this general chapter.

Assay responses can also be counts, such as number of plaques or colonies. Count responses are sometimes treated as quantitative, sometimes as qualitative, and sometimes models specific to integers are used. The choice is often based on the range of counts. If the count is mostly 0 and rarely greater than 1, the assay may be analyzed as quantal and the response is Any/None. If the counts are large and cover a wide range, such as 500 to 2500, then the assay may be analyzed as quantitative, possibly after transformation of the counts. A square root transformation of the count is often helpful in such analyses to better satisfy homogeneity of variances. If the range of counts includes or is near 0 but 0 is not the preponderant value, it may be preferable to use a model specific for integer responses. Poisson regression and negative binomial regression models are often good options. Models specific to integers will not be discussed further in this general chapter.

Assays with quantitative responses may be converted to quantal responses. For example, what may matter is whether some defined threshold is exceeded. The model could then be quantal—threshold exceeded or not. In general, assay systems have more precise estimates of potency if the model uses all the information in the response. Using above or below a threshold, rather than the measured quantitative responses, is likely to degrade the performance of an assay.

#### 3.2 Overview of Models for Quantitative Responses

In quantitative assays, the measurement is a number on a continuous scale. Optical density values from plate-based assays are such measurements. Models for quantitative assays can be linear or nonlinear. Although the two display an apparent difference in levels of complexity, parallel-line (linear) and parallel-curve (nonlinear) models share many commonalities. Because of the different form of the equations, slope-ratio assays are considered separately (section 3.3 Slope-Ratio Concentration–Response Models).

**ASSUMPTIONS**—The basic parallel-line, parallel-curve, and slope-ratio models share some assumptions. All include a residual term, e, that represents error (variability) which is assumed to be independent from measurement to measurement and to have constant variance from concentration to concentration and sample to sample. Often the residual term is assumed to have a normal distribution as well. The assumptions of independence and equal variances are commonly violated, so the goal in analysis is to incorporate the lack of independence and the unequal variances into the statistical model or the method of estimation.

Lack of independence often arises because of the design or conduct of the assay. For example, if the assay consists of responses from multiple plates, observations from the same plate are likely to share some common influence that is not shared with observations from other plates. This is an example of intraplate correlation. A simple approach for dealing with this lack of
independence is to include a block term in the statistical model for plate. With three or more plates this should be a random effects term so that we obtain an estimate of plate-to-plate variability.

In general, the model needs to closely reflect the design. The basic model equations given in sections 3.3–3.5 apply only to completely randomized designs. Any other design will mean additional terms in the statistical model. For example, if plates or portions of plates are used as blocks, one will need terms for blocks.

**Calculation of Potency**—A primary assumption underlying methods used for the calculation of relative potency is that of similarity. Two preparations are similar if they contain the same effective constituent or same effective constituents in the same proportions. If this condition holds, the Test preparation behaves as a dilution (or concentration) of the Standard preparation. Similarity can be represented mathematically as follows. Let $F_S$ be the concentration–response function for the Standard, and let $F_T$ be the concentration–response function for the Test. The underlying mathematical model for similarity is:

$$F_T(z) = F_S(\rho z), \quad [3.1]$$

where $z$ represents the concentration and $\rho$ represents the relative potency of the Test sample relative to the Standard sample.

Methods for estimating $\rho$ in some common concentration–response models are discussed below. For linear models, the distinction between parallel-line models (section 3.3 Parallel-Line Models for Quantitative Response) and slope-ratio models (section 3.5 Slope-Ratio Concentration–Response Models) is based on whether a straight-line fit to log concentration or concentration yields better agreement between the model and the data over the range of concentrations of interest.

### 3.3 Parallel-Line Models for Quantitative Responses

In this section, a linear model refers to a concentration–response relationship, which is a straight-line (linear) function between the logarithm of concentration, $x$, and the response, $y$. $y$ may be the response in the scale as measured or a transformation of the response. The functional form of this relationship is $y = \alpha + \beta x$. Straight-line fits may be used for portions of nonlinear concentration–response models, although doing so requires a method for selecting the concentrations to use for each of the Standard and Test samples (see (1032)).

**Means Model versus Regression**—A linear concentration–response model is most often analyzed with least squares regression. Such an analysis results in estimates of the unknown coefficients (intercepts and slope) and their standard errors, as well as measures of the goodness of fit [e.g., $R^2$ and root-mean-square error (RMSE)].

Linear regression works best where all concentrations can be used and there is negligible curvature in the concentration–response data. Another statistical method for analyzing linear concentration–response curves is the means model. This is an analysis of variance (ANOVA) method that offers some advantages, particularly when one or more concentrations from one or more samples are not used to estimate potency. Because a means model includes a separate mean for each unique combination of sample and dose (as well as block or other effects associated with the design structure) it is equivalent to a saturated polynomial regression model. Hence, a means model provides an estimate of error that is independent of regression lack of fit. In contrast, a regression residual based estimate of error is a mixture of the assay error, as estimated by the means model, combined with lack of fit of the regression model. At least in this sense, the means model error is a better estimate of the residual error variation in an assay system.

**Parallel-Line Concentration–Response Models**—If the general concentration–response model (3.1 Quantitative and Qualitative Assay Responses) can be made linear in $x = \log(z)$, the resulting equation is then:

$$y = \alpha + \beta \log(z) + e = \alpha + \beta x + e,$$

where $e$ is the residual or error term, and the intercept, $\alpha$, and slope, $\beta$, will differ between Test and Standard. With the parallelism (equal slopes) assumption, the model becomes

$$y_S = \alpha + \beta \log(z) + e = \alpha_S + \beta x + e \quad [3.2]$$

$$y_T = \alpha + \beta \log(z) + e = \alpha_T + \beta \log(\rho) + \beta x + e = \alpha_T + \beta \log(\rho) + \beta x + e,$$

where $S$ denotes Standard, $T$ denotes Test, $\alpha_S = \alpha$ is the y-intercept for the Standard, and $\alpha_T = \alpha + \beta \log(\rho)$ is the y-intercept for the Test (see Figure 3.1).
Figure 3.1. Example of parallel-line model.

Where concentration–response lines are parallel, as shown in Figure 3.1, a separation or horizontal shift indicates a difference in the level of biological activity being assayed. This horizontal difference is numerically \( \log(\rho) \), the logarithm of the relative potency, and is found as the vertical distance between the lines \( \alpha_T \) and \( \alpha_S \) divided by the slope, \( \beta \). The relative potency is then

\[
\rho = \text{antilog} \left( \frac{\alpha_T - \alpha_S}{\beta} \right)
\]

**ESTIMATION OF PARALLEL-LINE MODELS**—Parallel-line models are fit by the method of least squares. If the equal variance assumption holds, the parameters of equation [3.2] are chosen to minimize

\[
\hat{\rho} = \frac{\hat{\delta}}{\hat{\beta}}
\]

where the carets denote estimates. This is a linear regression with two independent variables, \( T \) and \( x \), where \( T \) is a variable that equals 1 for observations from the Test and 0 for observations from the Standard. The summation in equation [3.3] is over all observations of the Test and Standard. If the equal variance assumption does not hold but the variance is known to be inversely proportional to a value, \( w \), that does not depend on the current responses, the \( y \)'s, and can be determined for each observation, then the method is weighted least squares

\[
\sum w(y - \hat{\alpha}_S - \hat{\delta}T - \hat{\beta}x)^2
\]

Equation 3.4 is appropriate only if the weights are determined without using the response, the \( y \)'s, from the current data (see (1032) for guidance in determining weights). In equations [3.3] and [3.4] \( \hat{\delta} \) is the same as the \( \beta \) in equation [3.2] and \( \delta = \alpha_T - \alpha_S = \beta \log \rho \). So, the estimate of the relative potency, \( \rho \), is

\[
\hat{\rho} = \text{antilog} \left( \frac{\hat{\delta}}{\hat{\beta}} \right)
\]

Commonly available statistical software and spreadsheets provide routines for least squares. Not all software can provide weighted analyses.

See section 4 for methods to obtain a confidence interval for the estimated relative potency. For a confidence interval based on combining relative potency estimates from multiple assays, use the methods of section 4.2. For a confidence interval from a single assay, use Fieller's Theorem (section 4.3) applied to \( \hat{\delta}/\hat{\beta} \).

**MEASUREMENT OF NONPARALLELISM**—Parallelism for linear models is assessed by considering the difference or ratio of the two slopes. For the difference, this can be done by fitting the regression model,

\[
y = \alpha_s + \delta T + \beta_s x + \gamma x T + e
\]

where \( \delta = \alpha_T - \alpha_S \), \( \gamma = \beta_T - \beta_S \), and \( T = 1 \) for Test data and \( T = 0 \) for Standard data. Then use the standard t-distribution confidence interval for \( \gamma \). For the ratio of slopes, fit

\[
y = \alpha_s + \delta T + \beta_s (1 - T) + \beta_s x T + e
\]

and use Fieller's Theorem, equation [4.3], to obtain a confidence interval for \( \beta_T/\beta_S \).
3.4 Nonlinear Models for Quantitative Responses

Nonlinear concentration–response models are typically S-shaped functions. They occur when the range of concentrations is wide enough so that responses are constrained by upper and lower asymptotes. The most common of these models is the four-parameter logistic function as given below.

Let \( y \) denote the observed response and \( z \) the concentration. One form of the four-parameter logistic model is

\[
y = D + \frac{A - D}{1 + \left(\frac{z}{C}\right)^B} + e
\]

One alternative, but equivalent, form is

\[
y = a_0 + \frac{d}{1 + \text{antilog}\left(M\left(\log z - b\right)\right)} + e
\]

The two forms correspond as follows:
- Lower asymptote: \( D = a_0 \)
- Upper asymptote: \( A = a_0 + d \)
- Steepness: \( B = M \) (related to the slope of the curve at the \( EC_{50} \))
- Effective concentration 50% (\( EC_{50} \)): \( C = \text{antilog}(b) \) (may also be termed \( ED_{50} \)).

Any convenient base for logarithms is suitable; it is often convenient to work in log base 2, particularly when concentrations are twofold apart.

The four-parameter logistic curve is symmetric around the \( EC_{50} \) when plotted against log concentration because the rates of approach to the upper and lower asymptotes are the same (see Figure 3.2). For assays where this symmetry does not hold, asymmetrical model functions may be applied. These models are not considered further in this general chapter.

Figure 3.2. Examples of symmetric (four-parameter logistic) and asymmetric sigmoids.

In many assays the analyst has a number of strategic choices to make during assay development (see Development and Design of Biological Assays (1032)). For example, the responses could be modeled using a transformed response to a four-parameter logistic curve, or the responses could be weighted and fit to an asymmetric sigmoid curve. Also, it is often important to include terms in the model (often random effects) to address variation in the responses (or parameters of the response) associated with blocks or experimental units in the design of the assay. For simple assays where observations are independent, these strategic choices are fairly straightforward. For assays performed with grouped dilutions (as with multichannel pipets), assays with serial dilutions, or assay designs that include blocks (as with multiple plates per assay), it is usually a serious violation of the statistical assumptions to ignore the design structure. For such assays, a good approach involves a transformation that approximates a solution to non-constant variance, non-normality, and asymmetry combined with a model that captures the important parts of the design structure.

PARALLEL-CURVE CONCENTRATION-RESPONSE MODELS—The concept of parallelism is not restricted to linear models. For nonlinear curves, parallel or similar means the concentration–response curves can be superimposed following a horizontal displacement of one of the curves, as shown in Figure 3.3 for four-parameter logistic curves. In terms of the parameters of equation [3.5], this means the values of \( A, D, \) and \( B \) for the Test are the same as for the Standard.
Figure 3.3. Example of parallel curves from a nonlinear model.

The equations corresponding to the figure (with error term, e, added) are

\[
y_0 = D + \frac{A - D}{1 - \left(\frac{C}{C'}\right)^n} + e
\]

\[
y_1 = D + \frac{A - D}{1 - \left(\frac{C}{C'}\right)^n} + e
\]

or

\[
y_0 = D + \frac{A - D}{1 + \text{antilog} \left[ M(\log z - b) \right]} + e
\]

\[
y_1 = D + \frac{A - D}{1 + \text{antilog} \left[ M(\log z - b + \log \rho) \right]} + e
\]

Log \( \rho \) is the log of the relative potency and the horizontal distance between the two curves, just as for the parallel-line model. Because the EC\(_{50}\) of the standard is \( \text{antilog}(b) \) and that of the Test is \( \text{antilog}(b - \log \rho) = \text{antilog}(b)/\rho \), the relative potency is the ratio of EC\(_{50}\)'s (standard over Test) when the parallel-curve model holds.

ESTIMATION OF PARALLEL-CURVE MODELS—Estimation of nonlinear, parallel-curve models is similar to that for parallel-line models, possibly after transformation of the response and possibly with weighting. For the four-parameter logistic model, the parameter estimates are found by minimizing:

\[
\sum \left( \frac{y}{D} - \frac{A - D}{1 + \text{antilog} \left[ M(\log z - b + \log \rho) \right]} \right)^2
\]

without weighting, or

\[
\sum_w \left( \frac{y}{D} - \frac{A - D}{1 + \text{antilog} \left[ M(\log z - b + \log \rho) \right]} \right)^2
\]

with weighting. (As for equation [3.4], equation [3.6] is appropriate only if the weights are determined without using the responses, y's, from the current data.) In either case, the estimate of \( r \) is the estimate of the log of the relative potency. For some software, it may be easier to work with \( d = A - D \).

The parameters of the four-parameter logistic function and those of the asymmetric sigmoid models cannot be found with ordinary (linear) least squares regression routines. Computer programs with nonlinear estimation techniques must be used.

Analysts should not use the nonlinear regression fit to assess parallelism or estimate potency if any of the following are present:

a) inadequate asymptote information is available; or
b) a comparison of pooled error(s) from nonlinear regression to pooled error(s) from a means model shows that the nonlinear model does not fit well; or

Other appropriate measures of goodness of fit show that the nonlinear model is not appropriate (e.g., residual plots show evidence of a "hook").

See section 4 for methods to obtain a confidence interval for the estimated relative potency. For a confidence interval based on combining relative potency estimates from multiple assays, use the methods of section 4.2. For a confidence interval from a single assay, advanced techniques, such as likelihood profiles or bootstrapping are needed to obtain a confidence interval for the log relative potency, \( r \).
MEASUREMENT OF NONPARALLELISM—Assessment of parallelism for a four-parameter logistic model means assessing the slope parameter and the two asymptotes. During development (see (1032)), a decision should be made regarding which parameters are important and how to measure nonparallelism. As discussed in (1032), the measure of nonsimilarity may be a composite measure that considers all parameters together in a single measure, such as the parallelism sum of squares (see (1032)), or may consider each parameter separately. In the latter case, the measure may be functions of the parameters, such as an asymptote divided by the difference of asymptotes or the ratio of the asymptotes. For each parameter (or function of parameters), confidence intervals can be computed by bootstrap or likelihood profile methods. These methods are not presented in this general chapter.

3.5 Slope-Ratio Concentration–Response Models

If a straight-line regression fits the nontransformed concentration–response data well, a slope-ratio model may be used. The equations for the slope-ratio model assuming similarity are then:

\[
y_i = \alpha + \beta z + e = \alpha + \beta_S z + e \quad \text{[3.7]}
\]

\[
y_i = \alpha + \beta (\rho z) + e = \alpha + \beta_T z + e = \alpha + \beta_S z + e
\]

An identifying characteristic of a slope-ratio concentration–response model that can be seen in the results of a ranging study is that the lines for different potencies from a ranging study have the same intercept and different slopes. Thus, a graph of the ranging study resembles a fan. Figure 3.4 shows an example of a slope-ratio concentration–response model. Note that the common intercept need not be at the origin.

Figure 3.4. Example of slope-ratio model.

An assay with a slope-ratio concentration–response model for measuring relative potency consists, at a minimum, of one Standard sample and one Test sample, each measured at one or more concentrations and, usually, a measured response with no sample (zero concentration). Because the concentrations are not log transformed, they are typically equally spaced on the original, rather than log, scale. The model consists of one common intercept, a slope for the Test sample results, and a slope for the Standard sample results as in equation [3.7]. The relative potency is then found from the ratio of the slopes:

\[\text{Relative Potency} = \frac{\beta_T}{\beta_S} = \rho\]

ASSUMPTIONS FOR AND ESTIMATION OF SLOPE-RATIO MODELS—The assumptions for the slope-ratio model are the same as for parallel-line models: The residual terms are independent, have constant variance, and may need to have a normal distribution. The method of estimation is also least squares. This may be implemented either with or without weighting, as demonstrated in equations [3.8] and [3.9], respectively.

\[
\sum (y - \hat{\alpha} - \hat{\beta}_S z(1 - T) - \hat{\beta}_T z T)^2 \quad \text{[3.8]}
\]

\[
\sum w (y - \hat{\alpha} - \hat{\beta}_S z(1 - T) - \hat{\beta}_T z T)^2 \quad \text{[3.9]}
\]

Equation [3.9] is appropriate only if the weights are determined without using the response, the y’s, from the current data. This is a linear regression with two independent variables, z(1 − T) and zT, where T = 1 for Test data and T = 0 for Standard data. \(\hat{\beta}_T\) is the estimated slope for the Test, \(\hat{\beta}_S\) the estimated slope for the Standard, and then the estimate of relative potency is
Because the slope-ratio model is a linear regression model, most statistical packages and spreadsheets can be used to obtain the relative potency estimate. In some assay systems, it is sometimes appropriate to omit the zero concentration (e.g., if the no-dose controls are handled differently in the assay) and at times one or more of the high concentrations (e.g., if there is a hook effect where the highest concentrations do not have the highest responses). The discussion about using a means model and selecting subsets of concentrations for straight parallel-line bioassays applies to slope-ratio assays as well.

See section 4 for methods to obtain a confidence interval for the estimated relative potency. For a confidence interval based on combining relative potency estimates from multiple assays, use the methods of section 4.2. For a confidence interval from a single assay, use Fieller's Theorem (section 4.3) applied to

\[
R = \frac{\hat{\beta}_1}{\hat{\beta}_0}
\]

MEASUREMENT OF NONSIMILARITY—For slope-ratio models, statistical similarity corresponds to equal intercepts for the Standard and Test. To assess the similarity assumption it is necessary to have at least two nonzero concentrations for each sample. If the intercepts are not equal, equation \[3.7\] becomes

\[
y_S = \alpha_S + \beta_S z + e
\]

\[y_T = \alpha_T + \beta_T z + e\]

Departure from similarity is typically measured by the difference of intercepts, \(\alpha_T - \alpha_S\). An easy way to obtain a confidence interval is to fit the model,

\[
y = \alpha_S + \delta T + \beta_S z(1 - T) + \beta_T z T + e,
\]

where \(\delta = \alpha_T - \alpha_S\) and use the standard t-distribution-based confidence interval for \(\delta\).

### 3.6 Dichotomous (Quantal) Assays

For quantal assays the assay measurement has a dichotomous or binary outcome, e.g., in animal assays the animal is dead or alive or a certain physiologic response is or is not observed. For cellular assays, the quantal response may be whether there is or is not a response beyond some threshold in the cell. In cell-based viral titer or colony-forming assays, the quantal response may be a limit of integer response such as an integer number of particles or colonies. When one can readily determine if any particles are present—but not their actual number—then the assay can be analyzed as quantal. Note that if the reaction can be quantitated on a continuous scale, as with an optical density, then the assay is not quantal.

MODELS FOR QUANTAL ANALYSES—The key to models for quantal responses is to work with the probability of a response (e.g., probability of death), in contrast to quantitative responses for which the model is for the response itself. For each concentration, \(z\), a treated animal, as an example, has a probability of responding to that concentration, \(P(z)\). Often the curve \(P(z)\) can be approximated by a sigmoid when plotted against the logarithm of concentration, as shown in **Figure 3.5**. This curve shows that the probability of responding increases with concentration. The concentration that corresponds to a probability of 0.5 is the EC\(_{50}\).

![Figure 3.5. Example of sigmoid for \(P(z)\).](image)

The sigmoid curve is usually modeled based on the normal or logistic distribution. If the normal distribution is used, the resulting analysis is termed probit analysis, and if the logistic is used the analysis is termed logit or logistic analysis. The probit and logit models are practically indistinguishable, and either is an acceptable choice. The choice may be based on the availability of software that meets the laboratory’s analysis and reporting needs. Because software is more commonly available for logistic models (often under the term logistic regression) this discussion will focus on the use and interpretation of logit analysis. The considerations discussed in this section for logit analysis (using a logit transformation) apply as well to probit analysis (using a probit transformation).

**LOGIT MODEL**—The logit model for the probability of response, \(P(z)\), can be expressed in two equivalent forms. For the sigmoid,
where \( \log(ED_{50}) = -\frac{\beta_0}{\beta_1} \). An alternative form shows the relationship to linear models:

\[
\logit \text{ transform of } P = \log \left( \frac{P}{1-P} \right) = \beta_0 + \beta_1 \log(z) \quad [3.10]
\]

The log-linear form is usually shown using natural logs and is a useful reminder that many of the considerations, in particular linearity and parallelism, discussed for parallel-line models in section 3.3 Parallel-Line Models for Quantitative Responses apply to quantal models as well.

For a logit analysis with Standard and Test preparations, let \( T \) be a variable that takes the value 1 for animals receiving the Test preparation and 0 for animals receiving the Standard. Assuming parallelism of the Test and Standard curves, the logit model for estimating relative potency is then:

\[
\log \left( \frac{P}{1-P} \right) = \beta_0 + \beta_1 \log(z) + \beta_2 T
\]

The log of the relative potency of the Test compared to the Standard preparation is then \( \beta_2/\beta_1 \). The two curves in Figure 3.6 show parallel Standard and Test sigmoids. (If the corresponding linear forms equation [3.10] were shown, they would be two parallel straight lines.) The log of the relative potency is the horizontal distance between the two curves, in the same way as for the linear and four-parameter logistic models given for quantitative responses (sections 3.3 Parallel-Line Models for Quantitative Responses and 3.4 Nonlinear Models for Quantitative Responses).

**Figure 3.6. Example of Parallel Sigmoid Curves.**

**Estimating the Model Parameters and Relative Potency**—Two methods are available for estimating the parameters of logit and probit models: maximum likelihood and weighted least squares. The difference is not practically important, and the laboratory can accept the choice made by its software. The following assumes a general logistic regression software program. Specialized software should be similar.

Considering the form of equation [3.10], one observes a resemblance to linear regression. There are two independent variables, \( x = \log(z) \) and \( T \). For each animal, there is a yes/no dependent variable, often coded as 1 for yes or response and 0 for no or no response. Although bioassays are often designed with equal numbers of animals per concentration, that is not a requirement of analysis. Utilizing the parameters estimated by software, which include \( \beta_0, \beta_1, \) and \( \beta_2 \) and their standard errors, one obtains the estimate of the natural log of the relative potency:

\[
\text{Estimate of log of relative potency} = \frac{\hat{\beta}_2}{\hat{\beta}_1}
\]

See section 4 for methods to obtain a confidence interval for the estimated relative potency. For a confidence interval based on combining relative potency estimates from multiple assays, use the methods of section 4.2. For a confidence interval from a single assay, use Fieller’s Theorem (section 4.3) applied to \( \hat{\beta}_2/\hat{\beta}_1 \). The confidence interval for the relative potency is then [antilog(L), antilog(U)], where [L, U] is the confidence interval for the log relative potency.

**Assumptions**—Assumptions for quantal models have two parts. The first concerns underlying assumptions related to the probability of response of each animal or unit in the bioassay. These are difficult to verify assumptions that depend on the design of the assay. The second part concerns assumptions for the statistical model for \( P(z) \). Most important of these are parallelism and linearity. These assumptions can be checked much as for parallel-line analyses for quantitative responses.

In most cases, quantal analyses assume a standard binomial probability model, a common choice of distribution for dichotomous data. The key assumptions of the binomial are that at a given concentration each animal treated at that concentration has the same probability of responding and the results for any animal are independent from those of all other animals. This basic set of assumptions can be violated in many ways. Foremost among them is the presence of litter effects, where animals from the same litter tend to respond more alike than do animals from different litters. Cage effects, in which the environmental conditions or care rendered to any specific cage makes the animals from that cage more or less likely to respond to experimental treatment, violates the equal-probability and independence assumptions. These assumption violations and
4. CONFIDENCE INTERVALS

A report of an assay result should include a measure of the uncertainty of that result. This is often a standard error or a confidence interval. An interval (c, d), where c is the lower confidence limit and d is the upper confidence limit, is a 95% confidence interval for a parameter (e.g., relative potency) if 95% of such intervals upon repetition of the experiment would include the actual value of the parameter. A confidence interval may be interpreted as indicating values of the parameter that are consistent with the data. This interpretation of a confidence interval requires that various assumptions be satisfied. Assumptions also need to be satisfied when the width or half width [(d-c)/2] is used in a monograph as a measure of whether there is adequate precision to report a potency. The interval width is sometimes used as a suitability criterion without the confidence interpretation. In such cases the assumptions need not be satisfied.

Confidence intervals can either be model-based or sample-based. A model-based interval is based on the standard errors for each of the one or more estimates of log relative potency that come from the analysis of a particular statistical model. Model-based intervals should be avoided if sample-based intervals are possible. Model-based intervals require that the statistical model correctly incorporate all the effects and correlations that influence the model’s estimate of precision. These include but are not limited to serial dilution and plate effects. Section 4.3 Model-Based Methods describes Fieller’s Theorem, a commonly used model-based interval.

Sample-based methods combine independent estimates of log relative potency. Multiple assays may arise because this was determined to be required during development and validation or because the assay procedure fixes a maximum acceptable width of the confidence interval and two or more independent assays may be needed to meet the specified width requirement. Some sample-based methods do not require that the statistical model correctly incorporate all effects and correlations. However, this should not be interpreted as dismissing the value of addressing correlations and other factors that influence within-assay precision. The within-assay precision is used in similarity assessment and is a portion of the variability that is the basis for the sample-based intervals. Thus minimizing within-assay variability to the extent practical is important. Sample-based intervals are covered in section 4.2 Combining Independent Assays (Sample-Based Confidence Interval Methods).

4.1 Combining Results from Multiple Assays

In order to mitigate the effects of variability, it is appropriate to replicate independent bioassays and combine their results to obtain a single reportable value. That single reportable value (and not the individual assay results) is then compared to any applicable acceptance criteria. During assay development and validation, analysts should evaluate whether it is useful to combine the results of such assays and, if so, in what way to proceed.

There are two primary questions to address when considering how to combine results from multiple assays:

Are the assays mutually independent?
A set of assays may be regarded as mutually independent when the responses of one do not in any way depend on the distribution of responses of any of the others. This implies that the random errors in all essential factors influencing the result (for example, dilutions of the standard and of the preparation to be examined or the sensitivity of the biological indicator) in one assay must be independent of the corresponding random errors in the other assays. Assays on successive days using the original and retained dilutions of the Standard, therefore, are not independent assays. Similarly, if the responses, particularly the potency, depend on other reagents that are shared by assays (e.g., cell preparations), the assays may not be independent.

Assays need not be independent in order for analysts to combine results. However, methods for independent assays are much simpler. Also, combining dependent assay results may require assumptions about the form of the correlation between assay results that may be, at best, difficult to verify. Statistical methods are available for dependent assays, but they are not presented in this general chapter.

*Are the results of the assays homogeneous?*

Homogeneous results differ only because of random within-assay errors. Any contribution from factors associated with intermediate precision precludes homogeneity of results. Intermediate precision factors are those that vary between assays within a laboratory and can include analyst, equipment, and environmental conditions. There are statistical tests for heterogeneity, but lack of statistically significant heterogeneity is not properly taken as assurance of homogeneity and so no test is recommended. If analysts use a method that assumes homogeneity, homogeneity should be assessed during development, documented during validation, and monitored during ongoing use of the assay.

Additionally, before results from assays can be combined, analysts should consider the scale on which that combination is to be made. In general, the combination should be done on the scale for which the parameter estimates are approximately normally distributed. Thus, for relative potencies based on a parallel-line, parallel-curve, or quantal method, the relative potencies are combined in the logarithm scale.

### 4.2 Combining Independent Assays (Sample-Based Confidence Interval Methods)

Analysts can use several methods for combining the results of independent assays. A simple method described below (Method 1) assumes a common distribution of relative potencies across the assays and is recommended. A second procedure is provided and may be useful if homogeneity of relative potency across assays can be documented. A third alternative is useful if the assumptions for Methods 1 and 2 are not satisfied. Another alternative, analyzing all assays together using a linear or nonlinear mixed-effects model, is not discussed in this general chapter.

**METHOD 1—INDEPENDENT ASSAY RESULTS FROM A COMMON ASSAY DISTRIBUTION**—The following is a simple method that assumes independence of assays. It is assumed that the individual assay results (logarithms of relative potencies) are from a common normal distribution with some nonzero variance. This common distribution assumption requires that all assays to be combined use the same design and laboratory procedures. Implicit is that the relative potencies may differ between the assays. This method thus captures interassay variability in relative potency. Note that the individual relative potencies should not be rounded before combining results.

Let $R_i$ denote the logarithm of the relative potency of the $i$th assay of $N$ assay results to be combined. To combine the $N$ results, the mean, standard deviation, and standard error of the $R_i$ are calculated in the usual way:

$$\text{Mean } \bar{R} = \frac{\sum_{i=1}^{N} R_i}{N}$$

$$\text{Standard Deviation } S = \sqrt{\frac{1}{N-1} \sum_{i=1}^{N} (R_i - \bar{R})^2}$$

$$\text{Standard Error } SE = S / \sqrt{N}$$

A $100(1 - \alpha)$% confidence interval is then found as

$$\bar{R} \pm t_{N-1,\alpha/2} \times SE,$$

where $t_{N-1,\alpha/2}$ is the upper $\alpha/2$ percentage point of a $t$-distribution with $N-1$ degrees of freedom. The quantity $t_{N-1,\alpha/2} \times SE$ is the expanded uncertainty of $\bar{R}$. The number, $N$, of assays to be combined is usually small, and hence the value of $t$ is usually large. Because the results are combined in the logarithm scale, the combined result can be reported in the untransformed scale as a confidence interval for the geometric mean potency, estimated by $\text{antilog} (\bar{R})$,

$$\text{antilog}(\bar{R} - t_{N-1,\alpha/2} \times SE), \text{ antilog}(\bar{R} + t_{N-1,\alpha/2} \times SE)$$

**METHOD 2—INDEPENDENT ASSAY RESULTS, HOMOGENEITY ASSUMED**—This method can be used provided the following conditions are fulfilled:

1. The individual potency estimates form a homogeneous set with regard to the potency being estimated. Note that this means documenting (usually during development and validation) that there are no contributions to between-assay variability from intermediate precision factors. The individual results should appear to be consistent with homogeneity. In particular, differences between them should be consistent with their standard errors.
2. The potency estimates are derived from independent assays.
3. The number of degrees of freedom of the individual residual errors is not small. This is required so that the weights are well determined.
When these conditions are not fulfilled, this method cannot be applied and Method 1, Method 3, or some other method should be used. Further note that Method 2 (because it assumes no inter-assay variability) often results in narrower confidence intervals than Method 1, but this is not sufficient justification for using Method 2 absent satisfaction of the conditions listed above.

**Calculation of Weighting Coefficients**—It is assumed that the results of each of the N assays have been analyzed to give N estimates of log potency with associated confidence limits. For each assay, i, the logarithmic confidence interval for the log potency or log relative potency and a value $L_i$ are obtained by subtracting the lower confidence limit from the upper. (This formula, using the $L_i$, accommodates asymmetric confidence intervals such as from Fieller’s Theorem, section 4.3 Model-Based Methods). A weight $W_i$ for each value of the log relative potency, $R_i$, is calculated as follows, where $t_i$ has the same value as that used in the calculation of confidence limits in the $i$th assay:

$$W_i = \frac{4t_i^2}{L_i} \quad [4.1]$$

**Calculation of the Weighted Mean and Confidence Limits**—The products $WR_i$ are formed for each assay, and their sum is divided by the total weight for all assays to give the weighted mean log relative potency and its standard error as follows:

$$\text{Mean } R = \frac{\sum_{i=1}^{N} W_i R_i}{\sum_{i=1}^{N} W_i}$$

$$\text{Standard Error } SE = \frac{1}{\sqrt{\sum_{i=1}^{N} W_i}}$$

A 100(1 − α)% confidence interval in the log scale is then found as

$$R \pm t_{\alpha/2}, SE \quad [4.2]$$

where $t_{\alpha/2}$ is the upper $\alpha/2$ percentage point of a t-distribution with degrees of freedom, $k$, equal to the sum of the number of degrees of freedom for the error mean squares in the individual assays. This confidence interval can then be transformed back to the original scale as for Method 1.

**Method 3—Independent Assay Results, Common Assay Distribution NOT Assumed**—Method 3 is an approximate method that may be considered if the conditions for Method 1 (common assay distribution) or Method 2 (homogeneity) are not met.

The observed variation then has two components:

- the intra-assay variation for assay $i$:
  $$S_i^2 = 1/W_i$$

- the inter-assay variation:
  $$S^2 = \frac{1}{N-1} \sum_{i=1}^{N} (R_i - R)^2 - \frac{1}{N} \sum_{i=1}^{N} S_i^2$$

For each assay, a weighting coefficient is then calculated as

$$W_i^* = \frac{1}{S_i^2 + S^2}$$

which replaces $W_i$ in equation [4.1] and where $t$ in equation [4.2] is often approximated by the value 2.

### 4.3 Model-Based Methods

Many confidence intervals are of the form:

$$\text{Confidence interval} = \text{value} \pm k \times \text{standard error of that value}.$$ 

For such cases, as long as the multiplier $k$ can be easily determined (e.g., from a table of the t-distribution), reporting the standard error and the confidence interval are largely equivalent because the confidence interval is then easily determined from the standard error. However, the logarithms of relative potencies for parallel-line models and some parameterizations of nonlinear models and the relative potencies from slope-ratio models are ratios. In such cases, the confidence intervals are not symmetric around the estimated log relative potency or potency, and Fieller’s Theorem is needed. For these asymmetric cases the confidence interval should be reported because the standard error by itself does not capture the asymmetry.

Fieller’s Theorem is the formula for the confidence interval for a ratio. Let $R = a/b$ be the ratio for which we need a confidence interval. For the estimates of $a$ and $b$, we have their respective standard errors, $SE_a$ and $SE_b$, and a covariance between them, denoted $\text{Cov}$. (The covariance is a measure of the degree to which the estimates of $a$ and $b$ are related and is proportional to...
the correlation between the estimates of a and b.) The covariance may be 0, as for some parameterizations of standard parallel-line analyses, but it need not be. The confidence interval for R then is as follows:

\[
\frac{R - g \text{Cov}}{SE_a} \leq \frac{t}{b} \sqrt{(1 - g)SE_a^2 + \hat{R}^2SE_b^2 - 2\hat{R} \text{Cov}} \leq \frac{R + g \text{Cov}}{SE_a}
\]

where

\[
g = \frac{t \cdot SE_a}{b}
\]

and \( t \) is the appropriate t deviate value that will depend on the sample size and confidence level chosen (usually 95%). If \( g > 1 \), it means that the denominator, \( b \), is not statistically significantly different from 0 and the use of the ratio is not sensible for those data.

For those cases where the estimates of a and b are statistically uncorrelated (Cov = 0), the confidence interval formula simplifies to

\[
\frac{R \pm \frac{t}{b} \sqrt{(1 - g)SE_a^2 + \hat{R}^2SE_b^2}}{1 - g}
\]

5. ADDITIONAL SOURCES OF INFORMATION

A variety of statistical methods can be used to analyze bioassay data. This chapter presents several methods, but many other similar methods could also be employed. Additional information and alternative procedures can be found in the references listed below and other sources.


1041 BIOLOGICS

Products such as antitoxins, antivenins, blood, blood derivatives, immune serums, immunologic diagnostic aids, toxoids, vaccines, and related articles that are produced under license in accordance with the terms of the federal Public Health Service Act (58 Stat. 682) approved July 1, 1944, as amended, have long been known as “biologics.” However, in Table III, Part F, of the Act, the term “biological products” is applied to the group of licensed products as a whole. For Pharmacopeial purposes, the term “biologics” refers to those products that must be licensed under the Act and comply with Food and Drug Regulations—Code of Federal Regulations, Title 21 Parts 600-680, pertaining to federal control of these products (other than certain
diagnostic aids), as administered by the Center for Biologies Evaluation and Research or, in the case of the relevant diagnostic aids, by the Center for Devices and Radiological Health of the federal Food and Drug Administration.

Each lot of a licensed biologic is approved for distribution when it has been determined that the lot meets the specific control requirements for that product as set forth by the Office. Licensing includes approval of a specific series of production steps and in-process control tests as well as end-product specifications that must be met on a lot-by-lot basis. These can be altered only upon approval by the Center for Biologies Evaluation and Research and with the support of appropriate data demonstrating that the change will yield a final product having equal or superior safety, purity, potency, and efficacy. No lot of any licensed biological product is to be distributed by the manufacturer prior to the completion of the specified tests. Provisions generally applicable to biologic products include tests for potency, general safety, sterility, purity, water (residual moisture), pyrogens, identity, and constituent materials (Sections 610.10 to 610.15 and see Safety Tests—Biologicals under Biological Reactivity Tests, In Vivo (88), Sterility Tests (71), Water Determination (921), and Pyrogen Test (151), as well as Bacterial Endotoxins Test (85)). Constituent materials include ingredients, preservatives, diluents and adjuvants (which generally should meet compendial standards), extraneous protein in cell-culture produced vaccines (which, if other than serum-originating, is excluded) and antibiotics other than penicillin added to the production substrate of viral vaccines (for which compendial monographs on antibiotics and antibiotic substances are available). Additional specific safety tests are also required to be performed on live vaccines and certain other items. Where standard preparations are made available by the Center for Biologics Evaluation and Research (Section 610.20), such preparations are specified for comparison in potency or virulence testing. The U.S. Opacity Standard is used in estimating the bacterial concentration of certain bacterial vaccines and/or evaluating challenge cultures used in tests of them. (General Notices, 5.50.10 Units of Potency (Biological)).

The Pharmacopeial monographs conform to the Food and Drug Regulations in covering those aspects of identity, quality, purity, potency, and packaging and storage that are of particular interest to pharmacists and physicians responsible for the purchase, storage, and use of biologics. Revisions of the federal requirements affecting the USP monographs will be made the subjects of USP Supplements as promptly as practicable.

**VEHICLES AND ADDED SUBSTANCES**

Vehicles and added substances suitable for biologics are those named in the Food and Drug Regulations.

**CONTAINERS FOR INJECTIONS**

Containers for biologics intended to be administered by injection meet the requirements for Packaging and Storage Requirements (659), Injection Packaging.

**CONTAINER CONTENT**

The volumes in containers of biologics intended to be administered by injection meet the requirements for Container Content for Injections (697).

**LABELING**

Biologics intended to be administered by injection comply with the requirements for Labeling (7), Labels and Labeling for Injectable Products. In addition, the label on the final container for each biologic states the following: the title or proper name (the name under which the product is licensed under the Public Health Service Act); the name, address, and license number of the manufacturer; the lot number; the expiration date; and the recommended individual dose for multiple-dose containers. The package label includes all of the above, with the addition of the following: the preservative used and its amount; the number of containers, if more than one; the amount of product in the container; the recommended storage temperature; a statement, if necessary, that freezing is to be avoided; and such other information as the Food and Drug regulations may require.

**PACKAGING AND STORAGE**

The labeling gives the recommended storage temperature (see Packaging and Storage Requirements (659)). Precautions should be taken where products labeled to be stored at a temperature between 2° and 8° are stored in a refrigerator, in order to assure that they will not be frozen. Diluents packaged with biologics should not be frozen. Some products (as defined in Section 600.15) are to be maintained during shipment at specified temperatures.

**EXPIRATION DATE**

For compendial articles the expiration date identifies the time during which the article may be expected to meet the requirements of the Pharmacopeial monograph, provided it is kept under the prescribed storage conditions. This date limits the time during which the product may be dispensed or used (General Notices, 3.10 Applicability of Standards). However, for biological products, the stated date on each lot determines the dating period, which begins on the date of manufacture (Section 610.50) and beyond which the product cannot be expected beyond reasonable doubt to yield its specific results and to retain the required safety, purity, and potency (Section 300.3 (1) and (m)). Such a dating period may comprise an in-house storage period during which it is permitted to be held under prescribed conditions in the manufacturer’s storage, followed by a period...
QUALITY OF BIOTECHNOLOGICAL PRODUCTS: STABILITY TESTING OF BIOTECHNOLOGICAL/BIOLOGICAL PRODUCTS

INTRODUCTION (1)

The guidance stated in the ICH harmonized tripartite guideline entitled “Stability Testing of New Drug Substances and Products” (issued by ICH on October 27, 1993) applies in general to biotechnological/biological products. However, biotechnological/biological products have distinguishing characteristics to which consideration should be given in any well-defined testing program designed to confirm their stability during the intended storage period. For such products in which the active components are typically proteins and/or polypeptides, maintenance of molecular conformation and, hence, of biological activity, is dependent on noncovalent as well as covalent forces. The products are particularly sensitive to environmental factors such as temperature changes, oxidation, light, ionic content, and shear. To ensure maintenance of biological activity and to avoid degradation, stringent conditions for their storage are usually necessary.

The evaluation of stability may necessitate complex analytical methodologies. Assays for biological activity, where applicable, should be part of the pivotal stability studies. Appropriate physicochemical, biochemical, and immunochemical methods for the analysis of the molecular entity and the quantitative detection of degradation products should also be part of the stability program whenever purity and molecular characteristics of the product permit use of these methodologies.

With these concerns in mind, the applicant should develop the proper supporting stability data for a biotechnological/biological product and consider many external conditions that can affect the product’s potency, purity, and quality. Primary data to support a requested storage period for either drug substance or drug product should be based on long-term, real-time, real-condition stability studies. Thus, the development of a proper long-term stability program becomes critical to the successful development of a commercial product. The purpose of this document is to give guidance to applicants regarding the type of stability studies that should be provided in support of marketing applications. It is understood that during the review and evaluation process, continuing updates of initial stability data may occur.

SCOPE OF THE ANNEX (2)

The guidance stated in this annex to “Stability Testing of New Drug Substances and Products” applies to well-characterized proteins and polypeptides, their derivatives and products of which they are components, and which are isolated from tissues, body fluids, cell cultures, or produced using recombinant deoxyribonucleic acid (r-DNA) technology. Thus, the document covers the generation and submission of stability data for products such as cytokines (interferons, interleukins, colony-stimulating factors, tumor necrosis factors), erythropoietins, plasminogen activators, blood plasma factors, growth hormones and growth factors, insulin, monoclonal antibodies, and vaccines consisting of well-characterized proteins or polypeptides. In addition, the guidance outlined in the following sections may apply to other types of products, such as conventional vaccines, after consultation with the appropriate regulatory authorities. The document does not cover antibiotics, allergenic extracts, heparins, vitamins, whole blood, or cellular blood components.

TERMINOLOGY (3)

For the basic terms used in this annex, the reader is referred to the “Glossary” in “Stability Testing of New Drug Substances and Products.” However, because manufacturers of biotechnological/biological products sometimes use traditional terminology, traditional terms are specified in parentheses to assist the reader. A supplemental glossary is also included that explains certain terms used in the production of biotechnological/biological products.

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1 This guideline was developed within the Expert Working Group (Quality) of the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) and has been subject to consultation by the regulatory parties, in accordance with the ICH process. This document has been endorsed by the ICH Steering Committee at Step 4 of the ICH process, November 20, 1995. At Step 4 of the process, the final draft is recommended for adoption to the regulatory bodies of the European Union, Japan and the USA. This guideline was published in the Federal Register on July 10, 1996 (61 FR 36466) and is applicable to drug and biological products. Although this guideline does not create or confer any rights for or on any person and does not operate to bind FDA or the industry, it does represent the agency’s current thinking on stability testing of biotechnological/biological products. For additional copies of this guideline, contact the Drug Information Branch, HFD-210, CDER, FDA, 5600 Fishers Lane, Rockville, MD 20857 (Phone: 301-827-4573) or the Manufacturers Assistance and Communication Staff (HFM-42), CBER, FDA, 1401 Rockville Pike, Rockville, MD 20852-1448. Send one self-addressed adhesive label to assist the offices in processing your request. An electronic version of this guidance is also available via Internet using the World Wide Web (WWW) (connect to the CDER Home Page at http://www.fda.gov/cder and go to the “Regulatory Guidance” section).
SELECTED MATERIALS AND METHODS (4)

Drug Substance (Bulk Material) (4.1)

Where bulk material is to be stored after manufacture, but before formulation and final manufacturing, stability data should be provided at least three batches (which manufacture and storage are representative of the manufacturing scale of production). A minimum of 6 months' stability data at the time of submission should be submitted in cases where storage periods greater than 6 months are requested. For drug substances with storage periods of less than 6 months, the minimum amount of stability data in the initial submission should be determined on a case-by-case basis. Data from pilot-plant scale batches of drug substance produced at a reduced scale of fermentation and purification may be provided at the time the dossier is submitted to the regulatory agencies with an agreement to place the first three manufacturing scale batches into the long-term stability program after approval.

The quality of the batches of drug substance placed into the stability program should be representative of the quality of the material used in preclinical and clinical studies and of the quality of the material to be made at manufacturing scale. In addition, the drug substance (bulk material) made at pilot-plant scale should be produced by a process and stored under conditions representative of that used for the manufacturing scale. The drug substance entered into the stability program should be stored in containers that properly represent the actual holding containers used during manufacture. Containers of reduced size may be acceptable for drug substance stability testing provided that they are constructed of the same material and use the same type of container/closure system that is intended to be used during manufacture.

Intermediates (4.2)

During manufacture of biotechnological/biological products, the quality and control of certain intermediates may be critical to the production of the final product. In general, the manufacturer should identify intermediates and generate in-house data and process limits that assure their stability within the bounds of the developed process. Although the use of pilot-plant scale data is permissible, the manufacturer should establish the suitability of such data using the manufacturing scale process.

Drug Product (Final Container Product) (4.3)

Stability information should be provided on at least three batches of final container product representative of that which will be used at manufacturing scale. Where possible, batches of final container product included in stability testing should be derived from different batches of bulk material. A minimum of 6 months’ data at the time of submission should be submitted in cases where storage periods greater than 6 months are requested. For drug products with storage periods of less than 6 months, the minimum amount of stability data in the initial submission should be determined on a case-by-case basis. Product expiration dating should be based upon the actual data submitted in support of the application. Because dating is based upon the real-time/real-temperature data submitted for review, continuing updates of initial stability data should occur during the review and evaluation process. The quality of the final container product placed on stability studies should be representative of the quality of the material used in the preclinical and clinical studies. Data from pilot-plant scale batches of drug product may be provided at the time the dossier is submitted to the regulatory agencies with a commitment to place the first three manufacturing scale batches into the long-term stability program after approval. Where pilot-plant scale batches were submitted to establish the dating for a product and, in the event that the product produced at manufacturing scale does not meet those long-term stability specifications throughout the dating period or is not representative of the material used in preclinical and clinical studies, the applicant should notify the appropriate regulatory authorities to determine a suitable course of action.

Sample Selection (4.4)

Where one product is distributed in batches differing in fill volume (e.g., 1 milliliter (mL), 2 mL, or 10 mL), unitage (e.g., 10 units, 20 units, or 50 units), or mass (e.g., 1 milligram (mg), 2 mg, or 5 mg), samples to be entered into the stability program may be selected on the basis of a matrix system and/or by bracketing.

Matrixing, i.e., the statistical design of a stability study in which different fractions of samples are tested at different sampling points, should only be applied when appropriate documentation is provided that confirms that the stability of the samples tested represents the stability of all samples. The differences in the samples for the same drug product should be identified as, for example, covering different batches, different strengths, different sizes of the same closure, and, possibly, in some cases, different container/closure systems. Matrixing should not be applied to samples with differences that may affect stability, such as different strengths and different containers/closures, where it cannot be confirmed that the products respond similarly under storage conditions.

Where the same strength and exact container/closure system is used for three or more fill contents, the manufacturer may elect to place only the smallest and largest container size into the stability program, i.e., bracketing. The design of a protocol that incorporates bracketing assumes that the stability of the intermediate condition samples is represented by those at the extremes. In certain cases, data may be needed to demonstrate that all samples are properly represented by data collected for the extremes.
STABILITY-INDICATING PROFILE (5)

On the whole, there is no single stability-indicating assay or parameter that profiles the stability characteristics of a biotechnological/biological product. Consequently, the manufacturer should propose a stability-indicating profile that provides assurance that changes in the identity, purity, and potency of the product will be detected.

At the time of submission, applicants should have validated the methods that comprise the stability-indicating profile, and the data should be available for review. The determination of which tests should be included will be product-specific. The items emphasized in the following subsections are not intended to be all-inclusive, but represent product characteristics that should typically be documented to demonstrate product stability adequately.

Protocol (5.1)

The dossier accompanying the application for marketing authorization should include a detailed protocol for the assessment of the stability of both drug substance and drug product in support of the proposed storage conditions and expiration dating periods. The protocol should include all necessary information that demonstrates the stability of the biotechnological/biological product throughout the proposed expiration dating period including, for example, well-defined specifications and test intervals. The statistical methods that should be used are described in the tripartite guideline on stability.

Potency (5.2)

When the intended use of a product is linked to a definable and measurable biological activity, testing for potency should be part of the stability studies. For the purpose of stability testing of the products described in this guideline, potency is the specific ability or capacity of a product to achieve its intended effect. It is based on the measurement of some attribute of the product and is determined by a suitable in vivo or in vitro quantitative method. In general, potencies of biotechnological/biological products tested by different laboratories can be compared in a meaningful way only if expressed in relation to that of an appropriate reference material. For that purpose, a reference material calibrated directly or indirectly against the corresponding national or international reference material should be included in the assay.

Potency studies should be performed at appropriate intervals as defined in the stability protocol and the results should be reported in units of biological activity calibrated, whenever possible, against nationally or internationally recognized standards. Where no national or international reference standards exist, the assay results may be reported in in-house derived units using a characterized reference material.

In some biotechnological/biological products, potency is dependent upon the conjugation of the active ingredient(s) to a second moiety or binding to an adjuvant. Dissociation of the active ingredient(s) from the carrier used in conjugates or adjuvants should be examined in real-time/real-temperature studies (including conditions encountered during shipment). The assessment of the stability of such products may be difficult because, in some cases, in vitro tests for biological activity and physicochemical characterization are impractical or provide inaccurate results. Appropriate strategies (e.g., testing the product before conjugation/binding, assessing the release of the active compound from the second moiety, in vivo assays) or the use of an appropriate surrogate test should be considered to overcome the inadequacies of in vitro testing.

Purity and Molecular Characterization (5.3)

For the purpose of stability testing of the products described in this guideline, purity is a relative term. Because of the effect of glycosylation, deamidation, or other heterogeneities, the absolute purity of a biotechnological/biological product is extremely difficult to determine. Thus, the purity of a biotechnological/biological product should be typically assessed by more than one method and the purity value derived is method-dependent. For the purpose of stability testing, tests for purity should focus on methods for determination of degradation products.

The degree of purity, as well as the individual and total amounts of degradation products of the biotechnological/biological product entered into the stability studies, should be reported and documented whenever possible. Limits of acceptable degradation should be derived from the analytical profiles of batches of the drug substance and drug product used in the preclinical and clinical studies.

The use of relevant physicochemical, biochemical, and immunochemical analytical methodologies should permit a comprehensive characterization of the drug substance and/or drug product (e.g., molecular size, charge, hydrophobicity) and the accurate detection of degradation changes that may result from deamidation, oxidation, sulfoxidation, aggregation, or fragmentation during storage. As examples, methods that may contribute to this include electrophoresis (SDS-PAGE, immunoelectrophoresis, Western blot, isoelectrofocusing), high-resolution chromatography (e.g., reversed-phase chromatography, gel filtration, ion exchange, affinity chromatography), and peptide mapping.

Wherever significant qualitative or quantitative changes indicative of degradation product formation are detected during long-term, accelerated, and/or stress stability studies, consideration should be given to potential hazards and to the need for characterization and quantification of degradation products within the long-term stability program. Acceptable limits should be proposed and justified, taking into account the levels observed in material used in preclinical and clinical studies.

For substances that cannot be properly characterized or products for which an exact analysis of the purity cannot be determined through routine analytical methods, the applicant should propose and justify alternative testing procedures.

Other Product Characteristics (5.4)

The following product characteristics, though not specifically relating to biotechnological/biological products, should be monitored and reported for the drug product in its final container:
Visual appearance of the product (color and opacity for solutions/suspensions; color, texture, and dissolution time for powders), visible particulates in solutions or after the reconstitution of powders or lyophilized cakes, pH, and moisture level of powders and lyophilized products.

Sterility testing or alternatives (e.g., container/closure integrity testing) should be performed at a minimum initially and at the end of the proposed shelf life.

Additives (e.g., stabilizers, preservatives) or excipients may degrade during the dating period of the drug product. If there is any indication during preliminary stability studies that reaction or degradation of such materials adversely affect the quality of the drug product, these items may need to be monitored during the stability program.

The container/closure has the potential to affect the product adversely and should be carefully evaluated (see below).

**STORAGE CONDITIONS (6)**

**Temperature (6.1)**

Because most finished biotechnological/biological products need precisely defined storage temperatures, the storage conditions for the real-time/real-temperature stability studies may be confined to the proposed storage temperature.

**Humidity (6.2)**

Biotechnological/biological products are generally distributed in containers protecting them against humidity. Therefore, where it can be demonstrated that the proposed containers (and conditions of storage) afford sufficient protection against high and low humidity, stability tests at different relative humidities can usually be omitted. Where humidity-protecting containers are not used, appropriate stability data should be provided.

**Accelerated and Stress Conditions (6.3)**

As previously noted, the expiration dating should be based on real-time/real-temperature data. However, it is strongly suggested that studies be conducted on the drug substance and drug product under accelerated and stress conditions. Studies under accelerated conditions may provide useful support data for establishing the expiration date, provide product stability information or future product development (e.g., preliminary assessment of proposed manufacturing changes such as change in formulation, scale-up), assist in validation of analytical methods for the stability program, or generate information that may help elucidate the degradation profile of the drug substance or drug product. Studies under stress conditions may be useful in determining whether accidental exposures to conditions other than those proposed (e.g., during transportation) are deleterious to the product and also for evaluating which specific test parameters may be the best indicators of product stability. Studies of the exposure of the drug substance or drug product to extreme conditions may help to reveal patterns of degradation; if so, such changes should be monitored under proposed storage conditions. Although the tripartite guideline on stability describes the conditions of the accelerated and stress study, the applicant should note that those conditions may not be appropriate for biotechnological/biological products. Conditions should be carefully selected on a case-by-case basis.

**Light (6.4)**

Applicants should consult the appropriate regulatory authorities on a case-by-case basis to determine guidance for testing.

**Container/Closure (6.5)**

Changes in the quality of the product may occur due to the interactions between the formulated biotechnological/biological product and container/closure. Where the lack of interactions cannot be excluded in liquid products (other than sealed ampules), stability studies should include samples maintained in the inverted or horizontal position (i.e., in contact with the closure), as well as in the upright position, to determine the effects of the closure on product quality. Data should be supplied for all different container/closure combinations that will be marketed.

In addition to the standard data necessary for a conventional single-use vial, the applicant should demonstrate that the closure used with a multiple-dose vial is capable of withstanding the conditions of repeated insertions and withdrawals so that the product retains its full potency, purity, and quality for the maximum period specified in the instructions-for-use on containers, packages, and/or package inserts. Such labeling should be in accordance with relevant national/regional requirements.

**Stability after Reconstitution of Freeze-Dried Product (6.6)**

The stability of freeze-dried products after their reconstitution should be demonstrated for the conditions and the maximum storage period specified on containers, packages, and/or package inserts. Such labeling should be in accordance with relevant national/regional requirements.
TESTING FREQUENCY (7)

The shelf lives of biotechnological/biological products may vary from days to several years. Thus, it is difficult to draft uniform guidelines regarding the stability study duration and testing frequency that would be applicable to all types of biotechnological/biological products. With only a few exceptions, however, the shelf lives for existing products and potential future products will be within the range of 0.5 to 5 years. Therefore, the guidance is based upon expected shelf lives in that range. This takes into account the fact that degradation of biotechnological/biological products may not be governed by the same factors during different intervals of a long storage period.

When shelf lives of 1 year or less are proposed, the real-time stability studies should be conducted monthly for the first 3 months and at 3 month intervals thereafter. For products with proposed shelf lives of greater than 1 year, the studies should be conducted every 3 months during the first year of storage, every 6 months during the second year, and annually thereafter.

While the testing intervals listed above may be appropriate in the preapproval or prelicense stage, reduced testing may be appropriate after approval or licensure where data are available that demonstrate adequate stability. Where data exist that indicate the stability of a product is not compromised, the applicant is encouraged to submit a protocol that supports elimination of specific test intervals (e.g., 9-month testing) for postapproval/postlicensure, long-term studies.

SPECIFICATIONS (8)

Although biotechnological/biological products may be subject to significant losses of activity, physicochemical changes, or degradation during storage, international and national regulations have provided little guidance with respect to distinct release and end of shelf life specifications. Recommendations for maximum acceptable losses of activity, limits for physicochemical changes, or degradation during the proposed shelf life have not been developed for individual types or groups of biotechnological/biological products but are considered on a case-by-case basis. Each product should retain its specifications within established limits for safety, purity, and potency throughout its proposed shelf life. These specifications and limits should be derived from all available information using the appropriate statistical methods. The use of different specifications for release and expiration should be supported by sufficient data to demonstrate that the clinical performance is not affected, as discussed in the tripartite guideline on stability.

LABELING (9)

For most biotechnological/biological drug substances and drug products, precisely defined storage temperatures are recommended. Specific recommendations should be stated, particularly for drug substances and drug products that cannot tolerate freezing. These conditions, and where appropriate, recommendations for protection against light and/or humidity, should appear on containers, packages, and/or package inserts. Such labeling should be in accordance with relevant national and regional requirements.

GLOSSARY (10)

Conjugated Product: A conjugated product is made up of an active ingredient (e.g., peptide, carbohydrate) bound covalently or noncovalently to a carrier (e.g., protein, peptide, inorganic mineral) with the objective of improving the efficacy or stability of the product.

Degradation Product: A molecule resulting from a change in the drug substance (bulk material) brought about over time. For the purpose of stability testing of the products described in this guideline, such changes could occur as a result of processing or storage (e.g., by deamidation, oxidation, aggregation, proteolysis). For biotechnological/biological products, some degradation products may be active.

Impurity: Any component of the drug substance (bulk material) or drug product (final container product) that is not the chemical entity defined as the drug substance, an excipient, or other additives to the drug product.

Intermediate: For biotechnological/biological products, a material produced during a manufacturing process that is not the drug substance or the drug product but for which manufacture is critical to the successful production of the drug substance or the drug product. Generally, an intermediate will be quantifiable and specifications will be established to determine the successful completion of the manufacturing step before continuation of the manufacturing process. This includes material that may undergo further molecular modification or be held for an extended period before further processing.

Manufacturing Scale Production: Manufacture at the scale typically encountered in a facility intended for product production for marketing.

Pilot-Plant Scale: The production of the drug substance or drug product by a procedure fully representative of and simulating that to be applied at manufacturing scale. The methods of cell expansion, harvest, and product purification should be identical except for the scale of production.
VIRAL SAFETY EVALUATION OF BIOTECHNOLOGY PRODUCTS DERIVED FROM CELL LINES OF HUMAN OR ANIMAL ORIGIN

I. INTRODUCTION

This document is concerned with testing and evaluation of the viral safety of biotechnology products derived from characterized cell lines of human or animal origin (i.e., mammalian, avian, insect), and outlines data that should be submitted in the marketing application/registration package. For the purposes of this document, the term virus excludes nonconventional transmissible agents like those associated with Bovine Spongiform Encephalopathy (BSE) and scrapie. Applicants are encouraged to discuss issues associated with BSE with the regulatory authorities.

The scope of the document covers products derived from cell cultures initiated from characterized cell banks. It covers products derived from in vitro cell culture, such as interferons, monoclonal antibodies, and recombinant deoxyribonucleic acid (DNA)-derived products including recombinant subunit vaccines, and also includes products derived from hybridoma cells grown in vivo as ascites. In this latter case, special considerations apply and additional information on testing cells propagated in vivo is contained in Appendix 1. Inactivated vaccines, all live vaccines containing self-replicating agents, and genetically engineered live vectors are excluded from the scope of this document.

The risk of viral contamination is a feature common to all biotechnology products derived from cell lines. Such contamination could have serious clinical consequences and can arise from the contamination of the source cell lines themselves (cell substrates) or from adventitious introduction of virus during production. To date, however, biotechnology products derived from cell lines have not been implicated in the transmission of viruses. Nevertheless, it is expected that the safety of these products with regard to viral contamination can be reasonably assured only by the application of a virus testing program and assessment of virus removal and inactivation achieved by the manufacturing process, as outlined below.

Three principal, complementary approaches have evolved to control the potential viral contamination of biotechnology products:

1. Selecting and testing cell lines and other raw materials, including media components, for the absence of undesirable viruses which may be infectious and/or pathogenic for humans;
2. Assessing the capacity of the production processes to clear infectious viruses;
3. Testing the product at appropriate steps of production for absence of contaminating infectious viruses.

All testing suffers from the inherent limitation of quantitative virus assays, i.e., that the ability to detect low viral concentrations depends on statistical reasons on the size of the sample. Therefore, no single approach will necessarily establish the safety of a product. Confidence that infectious virus is absent from the final product will in many instances not be derived solely from direct testing for their presence, but also from a demonstration that the purification regimen is capable of removing and/or inactivating the viruses.

The type and extent of viral tests and viral clearance studies needed at different steps of production will depend on various factors and should be considered on a case-by-case and step-by-step basis. The factors that should be taken into account include the extent of cell bank characterization and qualification, the nature of any viruses detected, culture medium constituents, culture methods, facility and equipment design, the results of viral tests after cell culture, the ability of the process to clear viruses, and the type of product and its intended clinical use.

The purpose of this document is to describe a general framework for virus testing, experiments for the assessment of viral clearance, and a recommended approach for the design of viral tests and viral clearance studies. Related information is described in the appendices and selected definitions are provided in the glossary.

Manufacturers should adjust the recommendations presented here to their specific product and its production process. The approach used by manufacturers in their overall strategy for ensuring viral safety should be explained and justified. In addition to the detailed data that is provided, an overall summary of the viral safety assessment would be useful in facilitating the review by regulatory authorities. This summary should contain a brief description of all aspects of the viral safety studies and strategies used to prevent virus contamination as they pertain to this document.

II. POTENTIAL SOURCES OF VIRUS CONTAMINATION

Viral contamination of biotechnology products may arise from the original source of the cell lines or from adventitious introduction of virus during production processes.

A. Viruses That Could Occur in the Master Cell Bank (MCB)

Cells may have latent or persistent virus infection (e.g., herpesvirus) or endogenous retrovirus which may be transmitted vertically from one cell generation to the next, since the viral genome persists within the cell. Such viruses may be constitutively expressed or may unexpectedly become expressed as an infectious virus. Viruses can be introduced into the MCB by several routes such as: (1) Derivation of cell lines from infected animals; (2) use of virus to establish the cell line; (3) use of contaminated biological reagents such as animal serum components; (4) contamination during cell handling.

B. Adventitious Viruses That Could Be Introduced During Production

Adventitious viruses can be introduced into the final product by several routes including, but not limited to, the following:

1. Use of contaminated biological reagents such as animal serum components;
2. Use of a virus for the induction of expression
of specific genes encoding a desired protein; (3) use of a contaminated reagent, such as a monoclonal antibody affinity column; (4) use of a contaminated excipient during formulation; and (5) contamination during cell and medium handling. Monitoring of cell culture parameters can be helpful in the early detection of potential adventitious viral contamination.

III. CELL LINE QUALIFICATION: TESTING FOR VIRUSES

An important part of qualifying a cell line for use in the production of a biotechnology product is the appropriate testing for the presence of virus.

A. Suggested Virus Tests for MCB, Working Cell Bank (WCB) and Cells at the Limit of In Vitro Cell Age Used for Production

Table 1 shows examples of virus tests to be performed once only at various cell levels, including MCB, WCB, and cells at the limit of in vitro cell age used for production.

<table>
<thead>
<tr>
<th>Tests for Retroviruses and Other Endogenous Viruses</th>
<th>MCB</th>
<th>WCB</th>
<th>Cells at the Limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infectivity</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Electron microscopy</td>
<td>+1</td>
<td>–</td>
<td>+1</td>
</tr>
<tr>
<td>Reverse transcriptase</td>
<td>+4</td>
<td>–</td>
<td>+4</td>
</tr>
<tr>
<td>Other virus-specific tests</td>
<td>as appropriate</td>
<td>–</td>
<td>as appropriate</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Tests for Nonendogenous or Adventitious Viruses</th>
<th>MCB</th>
<th>WCB</th>
<th>Cells at the Limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>In vitro Assays</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>In vivo Assays</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Antibody production tests</td>
<td>+1</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Other virus-specific tests</td>
<td>+4</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

1 See text—section III.A.2.
2 Cells at the limit: Cells at the limit of in vitro cell age used for production (See text—section III.A.3.).
3 May also detect other agents.
4 Not necessary if positive by retrovirus infectivity test.
5 As appropriate for cell lines which are known to have been infected by such agents.
6 For the first WCB, this test should be performed on cells at the limit of in vitro cell age, generated from that WCB; for WCB’s subsequent to the first WCB, a single in vitro and in vivo test can be done either directly on the WCB or on cells at the limit of in vitro cell age.
7 e.g., MAP, RAP, HAP—usually applicable for rodent cell lines.
8 e.g., tests for cell lines derived from human, nonhuman primate, or other cell lines as appropriate.

1. MASTER CELL BANK

Extensive screening for both endogenous and nonendogenous viral contamination should be performed on the MCB. For heterohybrid cell lines in which one or more partners are human or nonhuman primate in origin, tests should be performed in order to detect viruses of human or nonhuman primate origin because viral contamination arising from these cells may pose a particular hazard.

Testing for nonendogenous viruses should include in vitro and in vivo inoculation tests and any other specific tests, including species-specific tests such as the mouse antibody production (MAP) test, that are appropriate, based on the passage history of the cell line, to detect possible contaminating viruses.

2. WORKING CELL BANK

Each WCB as a starting cell substrate for drug production should be tested for adventitious virus either by direct testing or by analysis of cells at the limit of in vitro cell age, initiated from the WCB. When appropriate nonendogenous virus tests have been performed on the MCB and cells cultured up to or beyond the limit of in vitro cell age have been derived from the WCB and used for testing for the presence of adventitious viruses, similar tests need not be performed on the initial WCB. Antibody production tests are usually not necessary for the WCB. An alternative approach in which full tests are carried out on the WCB rather than on the MCB would also be considered acceptable.

3. CELLS AT THE LIMIT OF IN VITRO CELL AGE USED FOR PRODUCTION

The limit of in vitro cell age used for production should be based on data derived from production cells expanded under pilot-plant scale or commercial-scale conditions to the proposed in vitro cell age or beyond. Generally, the production cells are obtained by expansion of the WCB; the MCB could also be used to prepare the production cells. Cells at the limit of in vitro cell age should be evaluated once for those endogenous viruses that may have been undetected in the MCB and WCB.
performance of suitable tests (e.g., in vitro and in vivo) at least once on cells at the limit of in vitro cell age used for production would provide further assurance that the production process is not prone to contamination by adventitious virus. If any adventitious viruses are detected at this level, the process should be carefully checked in order to determine the cause of the contamination, and should be completely redesigned if necessary.

B. Recommended Viral Detection and Identification Assays

Numerous assays can be used for the detection of endogenous and adventitious viruses. Table 2 outlines examples for these assays. They should be regarded as assay protocols recommended for the present, but the list is not all-inclusive or definitive. Since the most appropriate techniques may change with scientific progress, proposals for alternative techniques, when accompanied by adequate supporting data, may be acceptable. Manufacturers are encouraged to discuss these alternatives with the regulatory authorities. Other tests may be necessary depending on the individual case. Assays should include appropriate controls to ensure adequate sensitivity and specificity. Wherever a relatively high possibility of the presence of a specific virus can be predicted from the species of origin of the cell substrate, specific tests and/or approaches may be necessary. If the cell line used for production is of human or nonhuman primate origin, additional tests for human viruses, such as those causing immunodeficiency diseases and hepatitis, should be performed unless otherwise justified. The polymerase chain reaction (PCR) may be appropriate for detection of sequences of other human viruses as well as for other specific viruses. The following is a brief description of a general framework and philosophical background within which the manufacturer should justify what was done.

Table 2. Examples of the Use and Limitations of Assays Which May be Used to Test for Virus

<table>
<thead>
<tr>
<th>Test</th>
<th>Test Article</th>
<th>Detection Capability</th>
<th>Detection Limitation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibody production</td>
<td>Lysate of cells and their culture medium</td>
<td>Specific viral antigens</td>
<td>Antigens not infectious for animal test system</td>
</tr>
<tr>
<td>In vivo virus screen</td>
<td>Lysate of cells and their culture medium</td>
<td>Broad range of viruses pathogenic for humans</td>
<td>Agents failing to replicate or produce diseases in the test system</td>
</tr>
<tr>
<td>In vitro virus screen for:</td>
<td>Lysate of cells and their culture medium</td>
<td>Broad range of viruses pathogenic for humans</td>
<td>Agents failing to replicate or produce diseases in the test system</td>
</tr>
<tr>
<td>1. Cell bank characterization</td>
<td>1. Lysate of cells and their culture medium (for co-cultivation, intact cells should be in the test article)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. Production screen</td>
<td>2. Unprocessed bulk harvest or lysate of cells and their cell culture medium from the production reactor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TEM on:</td>
<td></td>
<td>Virus and virus-like particles</td>
<td>Qualitative assay with assessment of identity</td>
</tr>
<tr>
<td>1. Cell substrate</td>
<td>1. Viable cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. Cell culture supernatant</td>
<td>2. Cell-free culture supernatant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reverse transcriptase (RT)</td>
<td>Cell-free culture supernatant</td>
<td>Retroviruses and expressed retroviral RT</td>
<td>Only detects enzymes with optimal activity under preferred conditions. Interpretation may be difficult due to presence of cellular enzymes; background with some concentrated samples</td>
</tr>
<tr>
<td>Retrovirus (RV) infectivity</td>
<td>Cell-free culture supernatant</td>
<td>Infectious retroviruses</td>
<td>RV failing to replicate or form discrete foci or plaques in the chosen test system</td>
</tr>
<tr>
<td>Cocultivation</td>
<td>Viable cells</td>
<td>Infectious retroviruses</td>
<td>RV failing to replicate</td>
</tr>
<tr>
<td>1. Infectivity endpoint</td>
<td></td>
<td>1. See above under RV infectivity</td>
<td></td>
</tr>
<tr>
<td>2. TEM endpoint</td>
<td></td>
<td>2. See above under TEM¹</td>
<td></td>
</tr>
<tr>
<td>3. RT endpoint</td>
<td></td>
<td>3. See above under RT</td>
<td></td>
</tr>
<tr>
<td>PCR (Polymerase chain reaction)</td>
<td>Cells, culture fluid and other materials</td>
<td>Specific virus sequences</td>
<td>Primer sequences must be present. Does not indicate whether virus is infectious.</td>
</tr>
</tbody>
</table>

¹ In addition, difficult to distinguish test article from indicator cells.

1. TESTS FOR RETROVIRUSES

For the MCB and for cells cultured up to or beyond the limit of in vitro cell age used for production, tests for retroviruses, including infectivity assays in sensitive cell cultures and electron microscopy (EM) studies, should be carried out. If infectivity is not detected and no retrovirus or retrovirus-like particles have been observed by EM, reverse transcriptase (RT) or other appropriate assays should be performed to detect retroviruses that may be noninfectious. Induction studies have not been found to be useful.
2. IN VITRO ASSAYS

In vitro tests are carried out by the inoculation of a test article (see Table 2) into various susceptible indicator cell cultures capable of detecting a wide range of human and relevant animal viruses. The choice of cells used in the test is governed by the species of origin of the cell bank to be tested, but should include a human and/or a nonhuman primate cell line susceptible to human viruses. The nature of the assay and the sample to be tested are governed by the type of virus which may possibly be present based on the origin or handling of the cells. Both cytopathic and hemadsorbing viruses should be sought.

3. IN VIVO ASSAYS

A test article (see Table 2) should be inoculated into animals, including suckling and adult mice, and in embryonated eggs to reveal viruses that cannot grow in cell cultures. Additional animal species may be used, depending on the nature and source of the cell lines being tested. The health of the animals should be monitored and any abnormality should be investigated to establish the cause of the illness.

4. ANTIBODY PRODUCTION TESTS

Species-specific viruses present in rodent cell lines may be detected by inoculating test article (see Table 2) into virus-free animals and examining the serum antibody level or enzyme activity after a specified period. Examples of such tests are the mouse antibody production (MAP) test, rat antibody production (RAP) test, and hamster antibody production (HAP) test. The viruses currently screened for in the antibody production assays are discussed in Table 3.

Table 3. Virus Detected in Antibody Production Tests

<table>
<thead>
<tr>
<th>MAP</th>
<th>HAP</th>
<th>RAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ectromelia Virus</td>
<td>Lymphocytic Choriomeningitis Virus (LCM)</td>
<td>Hantaan Virus</td>
</tr>
<tr>
<td>Hantaan Virus</td>
<td>Pneumonia Virus of Mice (PVM)</td>
<td>Kilham Rat Virus (KRV)</td>
</tr>
<tr>
<td>K Virus</td>
<td>Reovirus Type 3 (Reo3)</td>
<td>Mouse Encephalomyelitis Virus (Theilers, GDVII)</td>
</tr>
<tr>
<td>Lactic Dehydrogenase Virus (LDM)</td>
<td>Sendai Virus</td>
<td>Pneumonia Virus of Mice (PVM)</td>
</tr>
<tr>
<td>Lymphocytic Choriomeningitis Virus (LCM)</td>
<td>SV5</td>
<td>Rat Coronavirus (RCV)</td>
</tr>
<tr>
<td>Minute Virus of Mice</td>
<td>Reovirus Type 3 (Reo3)</td>
<td></td>
</tr>
<tr>
<td>Mouse Adenovirus (MADV)</td>
<td>Sendai Virus</td>
<td></td>
</tr>
<tr>
<td>Mouse Cytomegalovirus (MCMV)</td>
<td>Sialoacryoadenitis Virus (SDAV)</td>
<td></td>
</tr>
<tr>
<td>Mouse Encephalomyelitis Virus (Theilers, GDVII)</td>
<td>Toolan Virus (HI)</td>
<td></td>
</tr>
<tr>
<td>Mouse Hepatitis Virus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse Rotavirus (EDIM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pneumonia Virus of Mice (PVM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Polyoma Virus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reovirus Type 3 (Reo3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sendai Virus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thymic Virus</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 Viruses for which there is evidence of capacity for infecting humans or primates.
2 Viruses for which there is no evidence of capacity for infecting humans.
3 Virus capable of replicating in vitro in cells of human or primate origin.

C. Acceptability of Cell Lines

It is recognized that some cell lines used for the manufacture of product will contain endogenous retroviruses, other viruses, or viral sequences. In such circumstances, the action plan recommended for manufacture is described in section V. of this document. The acceptability of cell lines containing viruses other than endogenous retroviruses will be considered on an individual basis by the regulatory authorities, by taking into account a risk/benefit analysis based on the benefit of the product and its intended clinical use, the nature of the contaminating viruses, their potential for infecting humans or for causing disease in humans, the purification process for the product (e.g., viral clearance evaluation data), and the extent of the virus tests conducted on the purified bulk.

IV. TESTING FOR VIRUSES IN UNPROCESSED BULK

The unprocessed bulk constitutes one or multiple pooled harvests of cells and culture media. When cells are not readily accessible (e.g., hollow fiber or similar systems), the unprocessed bulk would constitute fluids harvested from the fermenter. A representative sample of the unprocessed bulk, removed from the production reactor prior to further processing, represents
one of the most suitable levels at which the possibility of adventitious virus contamination can be determined with a high probability of detection. Appropriate testing for viruses should be performed at the unprocessed bulk level unless virus testing is made more sensitive by initial partial processing (e.g., unprocessed bulk may be toxic in test cell cultures, whereas partially processed bulk may not be toxic).

In certain instances, it may be more appropriate to test a mixture consisting of both intact and disrupted cells and their cell culture supernatants removed from the production reactor prior to further processing. Data from at least three lots of unprocessed bulk at pilot-plant scale or commercial scale should be submitted as part of the marketing application/registration package.

It is recommended that manufacturers develop programs for the ongoing assessment of adventitious viruses in production batches. The scope, extent, and frequency of virus testing on the unprocessed bulk should be determined by taking several points into consideration, including the nature of the cell lines used to produce the desired products, the results and extent of virus tests performed during the qualification of the cell lines, the cultivation method, raw material sources, and results of viral clearance studies. In vitro screening tests, using one or several cell lines, are generally employed to test unprocessed bulk. If appropriate, a PCR test or other suitable methods may be used.

Generally, harvest material in which adventitious virus has been detected should not be used to manufacture the product. If any adventitious viruses are detected at this level, the process should be carefully checked to determine the cause of the contamination, and appropriate actions taken.

V. RATIONALE AND ACTION PLAN FOR VIRAL CLEARANCE STUDIES AND VIRUS TESTS ON PURIFIED BULK

It is important to design the most relevant and rational protocol for virus tests from the MCB level, through the various steps of drug production, to the final product including evaluation and characterization of viral clearance from unprocessed bulk. The evaluation and characterization of viral clearance plays a critical role in this scheme. The goal should be to obtain the best reasonable assurance that the product is free of virus contamination.

In selecting viruses to use for a clearance study, it is useful to distinguish between the need to evaluate processes for their ability to clear viruses that are known to be present and the desire to estimate the robustness of the process by characterizing the clearance of nonspecific “model” viruses (described later). Definitions of “relevant,” specific, and nonspecific “model” viruses are given in the glossary. Process evaluation requires knowledge of how much virus may be present in the process, such as the unprocessed bulk, and how much can be cleared in order to assess product safety. Knowledge of the time dependence for inactivation procedures is helpful in ascertaining the effectiveness of the inactivation process. When evaluating clearance of known contaminants, in-depth, time-dependent inactivation studies, demonstration of reproducibility of inactivation/removal, and evaluation of process parameters should be provided. When a manufacturing process is characterized for robustness of clearance using nonspecific “model” viruses, particular attention should be paid to nonenveloped viruses in the study design. The extent of viral clearance characterization studies may be influenced by the results of tests on cell lines and unprocessed bulk. These studies should be performed as described in section VI. below.

Table 4 presents an example of an action plan in terms of process evaluation and characterization of viral clearance as well as virus tests on purified bulk, in response to the results of virus tests on cells and/or the unprocessed bulk. Various cases are considered. In all cases, characterization of viral clearance using nonspecific “model” viruses should be performed. The most common situations are Cases A and B. Production systems contaminated with a virus other than a rodent retrovirus are normally not used. Where there are convincing and well justified reasons for drug production using a cell line from Cases C, D, or E, these should be discussed with the regulatory authorities. With Cases C, D, and E, it is important to have validated effective steps to inactivate/remove the virus in question from the manufacturing process.

<table>
<thead>
<tr>
<th>Status</th>
<th>Case A</th>
<th>Case B</th>
<th>Case C²</th>
<th>Case D²</th>
<th>Case E²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Presence of virus¹</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(+)³</td>
</tr>
<tr>
<td>Virus-like particles¹</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(+)³</td>
</tr>
<tr>
<td>Retrovirus-like particles¹</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>(+)³</td>
</tr>
<tr>
<td>Virus identified</td>
<td>not applicable</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Virus pathogenic for humans</td>
<td>not applicable</td>
<td>-⁴</td>
<td>-⁴</td>
<td>+</td>
<td>unknown</td>
</tr>
<tr>
<td>Action</td>
<td>yes⁵</td>
<td>yes⁵</td>
<td>yes⁵</td>
<td>yes⁵</td>
<td>yes⁵</td>
</tr>
<tr>
<td>Process characterization of viral clearance using nonspecific “model” viruses</td>
<td>yes⁵</td>
<td>yes⁵</td>
<td>yes⁵</td>
<td>yes⁵</td>
<td>yes⁵</td>
</tr>
<tr>
<td>Process evaluation of viral clearance using “relevant” or specific “model” viruses</td>
<td>no</td>
<td>yes⁶</td>
<td>yes⁶</td>
<td>yes⁶</td>
<td>yes⁶</td>
</tr>
</tbody>
</table>

Table 4. Action Plan for Process Assessment of Viral Clearance and Virus Tests on Purified Bulk
Table 4. Action Plan for Process Assessment of Viral Clearance and Virus Tests on Purified Bulk (continued)

<table>
<thead>
<tr>
<th></th>
<th>Case A</th>
<th>Case B</th>
<th>Case C</th>
<th>Case D</th>
<th>Case E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test for virus in purified bulk</td>
<td>not applicable</td>
<td>yes³</td>
<td>yes³</td>
<td>yes³</td>
<td>yes³</td>
</tr>
</tbody>
</table>

1 Results of virus tests for the cell substrate and/or at the unprocessed bulk level. Cell cultures used for production which are contaminated with viruses will generally not be acceptable. Endogenous viruses (such as retroviruses) or viruses that are an integral part of the MCB may be acceptable if appropriate viral clearance evaluation procedures are followed.

2 The use of source material which is contaminated with viruses, whether or not they are known to be infectious and/or pathogenic in humans, will only be acceptable under very exceptional circumstances.

3 Virus has been observed by either direct or indirect methods.

4 Believed to be nonpathogenic.

5 Characterization of clearance using nonspecific “model” viruses should be performed.

6 Process evaluation for “relevant” viruses specific “model” viruses should be performed.

7 See text under Case E.

8 The absence of detectable virus should be confirmed for purified bulk by means of suitable methods having high specificity and sensitivity for the detection of the virus in question. For the purpose of marketing authorization, data from at least 3 lots of purified bulk manufactured at pilot-plant or commercial scale should be provided. However for cell lines such as CHO cells for which the endogenous particles have been extensively characterized and adequate clearance has been demonstrated, it is not usually necessary to assay for the presence of the noninfectious particles in purified bulk.

Case A

Where no virus, virus-like particle, or retrovirus-like particle has been demonstrated in the cells or in the unprocessed bulk, virus removal and inactivation studies should be performed with nonspecific “model” viruses as previously stated.

Case B

Where only a rodent retrovirus (or a retrovirus-like particle that is believed to be nonpathogenic, such as rodent A- and R-type particles) is present, process evaluation using a specific “model” virus, such as a murine leukemia virus, should be performed. Purified bulk should be tested using suitable methods having high specificity and sensitivity for the detection of the virus in question. For marketing authorization, data from at least three lots of purified bulk at pilot-plant scale or commercial scale should be provided. Cell lines such as Chinese hamster ovary (CHO), C127, baby hamster kidney (BHK), and murine hybridoma cell lines have frequently been used as substrates for drug production with no reported safety problems related to viral contamination of the products. For these cell lines in which the endogenous particles have been extensively characterized and clearance has been demonstrated, it is not usually necessary to assay for the presence of the noninfectious particles in purified bulk. Studies with nonspecific “model” viruses, as in Case A, are appropriate.

Case C

When the cells or unprocessed bulk are known to contain a virus, other than a rodent retrovirus, for which there is no evidence of capacity for infecting humans (such as those identified by footnote 2 in Table 3, except rodent retroviruses (Case B)), virus removal and inactivation evaluation studies should use the identified virus. If it is not possible to use the identified virus, “relevant” or specific “model” viruses should be used to demonstrate acceptable clearance. Time-dependent inactivation for identified (or “relevant” or specific “model”) viruses at the critical inactivation step(s) should be obtained as part of process evaluation for these viruses. Purified bulk should be tested using suitable methods having high specificity and sensitivity for the detection of the virus in question. For the purpose of marketing authorization, data from at least three lots of purified bulk manufactured at pilot-plant scale or commercial scale should be provided.

Case D

Where a known human pathogen, such as those indicated by footnote 1 in Table 3, is identified, the product may be acceptable only under exceptional circumstances. In this instance, it is recommended that the identified virus be used for virus removal and inactivation evaluation studies and specific methods with high specificity and sensitivity for the detection of the virus in question be employed. If it is not possible to use the identified virus, “relevant” and/or specific “model” viruses (described later) should be used. The process should be shown to achieve the removal and inactivation of the selected viruses during the purification and inactivation processes. Time-dependent inactivation data for the critical inactivation step(s) should be obtained as part of process evaluation. Purified bulk should be tested using suitable methods having high specificity and sensitivity for the detection of the virus in question. For the purpose of marketing authorization, data from at least three lots of purified bulk manufactured at pilot-plant scale or commercial scale should be provided.

Case E

When a virus that cannot be classified by currently available methodologies is detected in the cells or unprocessed bulk, the product is usually considered unacceptable since the virus may prove to be pathogenic. In the very rare case where there are convincing and well justified reasons for drug production using such a cell line, this should be discussed with the regulatory authorities before proceeding further.
VI. EVALUATION AND CHARACTERIZATION OF VIRAL CLEARANCE PROCEDURES

Evaluation and characterization of due virus removal and/or inactivation procedures play an important role in establishing the safety of biotechnology products. Many instances of contamination in the past have occurred with agents whose presence was not known or even suspected, and though this happened to biological products derived from various source materials other than fully characterized cell lines, assessment of viral clearance will provide a measure of confidence that any unknown, unsuspected, and harmful viruses may be removed. Studies should be carried out in a manner that is well documented and controlled.

The objective of viral clearance studies is to assess process step(s) that can be considered to be effective in inactivating/removing viruses and to estimate quantitatively the overall level of virus reduction obtained by the process. This should be achieved by the deliberate addition (“spiking”) of significant amounts of a virus to the crude material and/or to different fractions obtained during the various process steps and demonstrating its removal or inactivation during the subsequent steps. It is not considered necessary to evaluate or characterize every step of a manufacturing process if adequate clearance is demonstrated by the use of fewer steps. It should be borne in mind that other steps in the process may have an indirect effect on the viral inactivation/removal achieved. Manufacturers should explain and justify the approach used in studies for evaluating virus clearance.

The reduction of virus infectivity may be achieved by removal of virus particles or by inactivation of viral infectivity. For each production step assessed, the possible mechanism of loss of viral infectivity should be described with regard to whether it is due to inactivation or removal. For inactivation steps, the study should be planned in such a way that samples are taken at different times and an inactivation curve constructed (see section VI.B.5.).

Viral clearance evaluation studies are performed to demonstrate the clearance of a virus known to be present in the MCB and/or to provide some level of assurance that adventitious viruses which could not be detected, or might gain access to the production process, would be cleared. Reduction factors are normally expressed on a logarithmic scale, which implies that, while residual virus infectivity will never be reduced to zero, it may be greatly reduced mathematically.

In addition to clearance studies for viruses known to be present, studies to characterize the ability to remove and/or inactivate other viruses should be conducted. The purpose of studies with viruses exhibiting a range of biochemical and biophysical properties that are not known or expected to be present is to characterize the robustness of the procedure rather than to achieve a specific inactivation or removal goal. A demonstration of the capacity of the production process to inactivate or remove viruses is desirable (see section VI.C.). Such studies are not performed to evaluate a specific safety risk. Therefore, a specific clearance value need not be achieved.

A. The Choice of Viruses for the Evaluation and Characterization of Viral Clearance

Viruses for clearance evaluation and process characterization studies should be chosen to resemble viruses which may contaminate the product and to represent a wide range of physico-chemical properties in order to test the ability of the system to eliminate viruses in general. The manufacturer should justify the choice of viruses in accordance with the aims of the evaluation and characterization study and the guidance provided in this document.

1. “RELEVANT” VIRUSES AND “MODEL” VIRUSES

A major issue in performing a viral clearance study is to determine which viruses should be used. Such viruses fall into three categories: “Relevant” viruses, specific “model” viruses, and nonspecific “model” viruses.

“Relevant” viruses are viruses used in process evaluation of viral clearance studies which are either the identified viruses, or of the same species as the viruses that are known, or likely to contaminate the cell substrate or any other reagents or materials used in the production process. The purification and/or inactivation process should demonstrate the capability to remove and/or inactivate such viruses. When a “relevant” virus is not available or when it is not well adapted to process evaluation of viral clearance studies (e.g., it cannot be grown in vitro to sufficiently high titers), a specific “model” virus should be used as a substitute. An appropriate specific “model” virus may be a virus which is closely related to the known or suspected virus (same genus or family), having similar physical and chemical properties to the observed or suspected virus.

Cell lines derived from rodents usually contain endogenous retrovirus particles or retrovirus-like particles, which may be infectious (C-type particles) or noninfectious (cytoplasmic A- and R-type particles). The capacity of the manufacturing process to remove and/or inactivate rodent retroviruses from products obtained from such cells should be determined. This may be accomplished by using a murine leukemia virus, a specific “model” virus in the case of cells of murine origin. When human cell lines secreting monoclonal antibodies have been obtained by the immortalization of B lymphocytes by Epstein-Barr Virus (EBV), the ability of the manufacturing process to remove and/or inactivate a herpes virus should be determined. Pseudorabies virus may also be used as a specific “model” virus.

When the purpose is to characterize the capacity of the manufacturing process to remove and/or inactivate viruses in general, i.e., to characterize the robustness of the clearance process, viral clearance characterization studies should be performed with nonspecific “model” viruses with differing properties. Data obtained from studies with “relevant” and/or specific “model” viruses may also contribute to this assessment. It is not necessary to test all types of viruses. Preference should be given to viruses that display a significant resistance to physical and/or chemical treatments. The results obtained for such viruses provide useful information about the ability of the production process to remove and/or inactivate viruses in general. The choice and number of viruses used will be influenced by the quality and characterization of the cell lines and the production process.

Examples of useful “model” viruses representing a range of physico-chemical structures and examples of viruses which have been used in viral clearance studies are given in Appendix 2 and Table A-1.
2. OTHER CONSIDERATIONS

Additional points to be considered are as follows:
(a) Viruses which can be grown to high titer are desirable, although this may not always be possible.
(b) There should be an efficient and reliable assay for the detection of each virus used, for every stage of manufacturing that is tested.
(c) Consideration should be given to the health hazard which certain viruses may pose to the personnel performing the clearance studies.

B. Design and Implications of Viral Clearance Evaluation and Characterization Studies

1. FACILITY AND STAFF

It is inappropriate to introduce any virus into a production facility because of good manufacturing practice (GMP) constraints. Therefore, viral clearance studies should be conducted in a separate laboratory equipped for virological work and performed by staff with virological expertise in conjunction with production personnel involved in designing and preparing a scaled-down version of the purification process.

2. SCALED-DOWN PRODUCTION SYSTEM

The validity of the scaling down should be demonstrated. The level of purification of the scaled-down version should represent as closely as possible the production procedure. For chromatographic equipment, column bed-height, linear flow-rate, flow-rate-to-bed-volume ratio (i.e., contact time), buffer and gel types, pH, temperature, and concentration of protein, salt, and product should all be shown to be representative of commercial-scale manufacturing. A similar elution profile should result. For other procedures, similar considerations apply. Deviations that cannot be avoided should be discussed with regard to their influence on the results.

3. ANALYSIS OF STEP-WISE ELIMINATION OF VIRUS

When viral clearance studies are being performed, it is desirable to assess the contribution of more than one production step to virus elimination. Steps which are likely to clear virus should be individually assessed for their ability to remove and inactivate virus and careful consideration should be given to the exact definition of an individual step. Sufficient virus should be present in the material of each step to be tested so that an adequate assessment of the effectiveness of each step is obtained. Generally, virus should be added to in-process material of each step to be tested. In some cases, simply adding high titer virus to unpurified bulk and testing its concentration between steps will be sufficient. Where virus removal results from separation procedures, it is recommended that, if appropriate and if possible, the distribution of the virus load in the different fractions be investigated. When virucidal buffers are used in multiple steps within the manufacturing process, alternative strategies such as parallel spiking in less virucidal buffers may be carried out as part of the overall process assessment. The virus titer before and after each step being tested should be determined. Quantitative infectivity assays should have adequate sensitivity and reproducibility and should be performed with sufficient replicates to ensure adequate statistical validity of the result. Quantitative assays not associated with infectivity may be used if justified. Appropriate virus controls should be included in all infectivity assays to ensure the sensitivity of the method. Also, the statistics of sampling virus when at low concentrations should be considered (Appendix 4).

4. DETERMINING PHYSICAL REMOVAL VERSUS INACTIVATION

Reduction in virus infectivity may be achieved by the removal or inactivation of virus. For each production step assessed, the possible mechanism of loss of viral infectivity should be described with regard to whether it is due to inactivation or removal. If little clearance of infectivity is achieved by the production process, and the clearance of virus is considered to be a major factor in the safety of the product, specific or additional inactivation/removal steps should be introduced. It may be necessary to distinguish between removal and inactivation for a particular step, for example, when there is a possibility that a buffer used in more than one clearance step may contribute to inactivation during each step, i.e., the contribution to inactivation by a buffer shared by several chromatographic steps and the removal achieved by each of these chromatographic steps should be distinguished.

5. INACTIVATION ASSESSMENT

For assessment of viral inactivation, unprocessed crude material or intermediate material should be spiked with infectious virus and the reduction factor calculated. It should be recognized that virus inactivation is not a simple, first order reaction and is usually more complex, with a fast “phase 1” and a slow “phase 2.” The study should, therefore, be planned in such a way that samples are taken at different times and an inactivation curve constructed. It is recommended that studies for inactivation include at least one time point less than the minimum exposure time and greater than zero, in addition to the minimum exposure time. Additional data are particularly important where the virus is a “relevant” virus known to be a human pathogen and an effective inactivation process is being designed. However, for inactivation studies in which nonspecific “model” viruses are used or when specific “model” viruses are used as surrogates for virus particles, such as the CHO intracytoplasmic retrovirus-like particles, reproducible clearance should be demonstrated in at least two independent studies. Whenever possible, the initial virus load should be determined from the virus that can be detected in the spiked starting material. If this is not possible, the initial virus load may be calculated from the titer of the spiking virus preparation. Where inactivation is too rapid
to plot an inactivation curve using process conditions, appropriate controls should be performed to demonstrate that infectivity is indeed lost by inactivation.

6. FUNCTION AND REGENERATION OF COLUMNS

Over time and after repeated use, the ability of chromatography columns and other devices used in the purification scheme to clear virus may vary. Some estimate of the stability of the viral clearance after several uses may provide support for repeated use of such columns. Assurance should be provided that any virus potentially retained by the production system would be adequately destroyed or removed prior to reuse of the system. For example, such evidence may be obtained by demonstrating that the cleaning and regeneration procedures do inactivate or remove virus.

7. SPECIFIC PRECAUTIONS

(a) Care should be taken in preparing the high-titer virus to avoid aggregation which may enhance physical removal and decrease inactivation, thus distorting the correlation with actual production.
(b) Consideration should be given to the minimum quantity of virus which can be reliably assayed.
(c) The study should include parallel control assays to assess the loss of infectivity of the virus due to such reasons as the dilution, concentration, filtration or storage of samples before titration.
(d) The virus “spike” should be added to the product in a small volume so as not to dilute or change the characteristics of the product. Diluted, test-protein sample is no longer identical to the product obtained at commercial scale.
(e) Small differences in, for example, buffers, media, or reagents can substantially affect viral clearance.
(f) Virus inactivation is time-dependent; therefore, the amount of time a spiked product remains in a particular buffer solution or on a particular chromatography column should reflect the conditions of the commercial-scale process.
(g) Buffers and product should be evaluated independently for toxicity or interference in assays used to determine the virus titer, as these components may adversely affect the indicator cells. If the solutions are toxic to the indicator cells, dilution, adjustment of the pH, or dialysis of the buffer containing spiked virus might be necessary. If the product itself has anti-viral activity, the clearance study may need to be performed without the product in a “mock” run, although omitting the product or substituting a similar protein that does not have anti-viral activity could affect the behavior of the virus in some production steps. Sufficient controls to demonstrate the effect of procedures used solely to prepare the sample for assay (e.g., dialysis, storage) on the removal/inactivation of the spiked virus should be included.
(h) Many purification schemes use the same or similar buffers or columns repetitively. The effects of this approach should be taken into account when analyzing the data. The effectiveness of virus elimination by a particular process may vary with the manufacturing stage at which it is used.
(i) Overall reduction factors may be underestimated where production conditions or buffers are too cytotoxic or virucidal and should be discussed on a case-by-case basis. Overall reduction factors may also be overestimated due to inherent limitations or inadequate design of viral clearance studies.

C. Interpretation of Viral Clearance Studies; Acceptability

The object of assessing virus inactivation/removal is to evaluate and characterize process steps that can be considered to be effective in inactivating/removing viruses and to estimate quantitatively the overall level of virus reduction obtained by the manufacturing process. For virus contaminants, as in Cases B through E, it is important to show that not only is the virus eliminated or inactivated, but that there is excess capacity for viral clearance built into the purification process to assure an appropriate level of safety for the final product. The amount of virus eliminated or inactivated by the production process should be compared to the amount of virus which may be present in unprocessed bulk.

To carry out this comparison, it is important to estimate the amount of virus in the unprocessed bulk. This estimate should be obtained using assays for infectivity or other methods such as transmission electron microscopy (TEM). The entire purification process should be able to eliminate substantially more virus than is estimated to be present in a single-dose-equivalent of unprocessed bulk. See Appendix 5 for calculation of virus reduction factors and Appendix 6 for calculation of estimated particles per dose.

Manufacturers should recognize that clearance mechanisms may differ between virus classes. A combination of factors should be considered when judging the data supporting the effectiveness of virus inactivation/removal procedures. These include:
(i) The appropriateness of the test viruses used;
(ii) The design of the clearance studies;
(iii) The log reduction achieved;
(iv) The time dependence of inactivation;
(v) The potential effects of variation in process parameters on virus inactivation/removal;
(vi) The limits of assay sensitivities;
(vii) The possible selectivity of inactivation/removal procedure(s) for certain classes of viruses.

Effective clearance may be achieved by any of the following: Multiple inactivation steps, multiple complementary separation steps, or combinations of inactivation and separation steps. Since separation methods may be dependent on the extremely specific physico-chemical properties of a virus which influence its interaction with gel matrices and precipitation properties, “model” viruses may be separated in a different manner than a target virus. Manufacturing parameters influencing separation should be properly defined and controlled. Differences may originate from changes in surface properties such as glycosylation. However, despite these potential variables, effective removal can be obtained by a combination of complementary separation steps or combinations of inactivation and separation steps. Therefore, well-defined separation steps, such as chromatographic procedures, filtration steps, and extractions, can be effective virus removal steps provided that they are performed under appropriately controlled conditions. An effective virus removal step should give reproducible reduction of virus load shown by at least two independent studies.
An overall reduction factor is generally expressed as the sum of the individual factors. However, reduction in virus titer of the order of 1 log10 or less would be considered negligible and would be ignored unless justified.

If little reduction of infectivity is achieved by the production process, and the removal of virus is considered to be a major factor in the safety of the product, a specific, additional inactivation/removal step or steps should be introduced. For all viruses, manufacturers should justify the acceptability of the reduction factors obtained. Results would be evaluated on the basis of the factors listed above.

D. Limitations of Viral Clearance Studies

Viral clearance studies are useful for contributing to the assurance that an acceptable level of safety in the final product is achieved but do not by themselves establish safety. However, a number of factors in the design and execution of viral clearance studies may lead to an incorrect estimate of the ability of the process to remove virus infectivity. These factors include the following:

1. Virus preparations used in clearance studies for a production process are likely to be produced in tissue culture. The behavior of a tissue culture virus in a production step may be different from that of the native virus, for example, if native and cultured viruses differ in purity or degree of aggregation.

2. Inactivation of virus infectivity frequently follows a biphasic curve in which a rapid initial phase is followed by a slower phase. It is possible that virus escaping a first inactivation step may be more resistant to subsequent steps. For example, if the resistant fraction takes the form of virus aggregates, infectivity may be resistant to a range of different chemical treatments and to heating.

3. The ability of the overall process to remove infectivity is expressed as the sum of the logarithm of the reductions at each step. The summation of the reduction factors of multiple steps, particularly of steps with little reduction (e.g., below 1 log10), may overestimate the true potential for virus elimination. Furthermore, reduction values achieved by repetition of identical or near identical procedures should not be included unless justified.

4. The expression of reduction factors as logarithmic reductions in titer implies that, while residual virus infectivity may be greatly reduced, it will never be reduced to zero. For example, a reduction in the infectivity of a preparation containing 8 log10 infectious units per milliliter (mL) by a factor of 8 log10 leaves zero log10 per mL or one infectious unit per mL, taking into consideration the limit of detection of the assay.

5. Pilot-plant scale processing may differ from commercial-scale processing despite care taken to design the scaled-down process.

6. Addition of individual virus reduction factors resulting from similar inactivation mechanisms along the manufacturing process may overestimate overall viral clearance.

E. Statistics

The viral clearance studies should include the use of statistical analysis of the data to evaluate the results. The study results should be statistically valid to support the conclusions reached (see Appendix 3).

F. Reevaluation of Viral Clearance

Whenever significant changes in the production or purification process are made, the effect of that change, both direct and indirect, on viral clearance should be considered and the system re-evaluated as needed. For example, changes in production processes may cause significant changes in the amount of virus produced by the cell line; changes in process steps may change the extent of viral clearance.

VII. SUMMARY

This document suggests approaches for the evaluation of the risk of viral contamination and for the removal of virus from product, thus contributing to the production of safe biotechnology products derived from animal or human cell lines, and emphasizes the value of many strategies, including:

A. Thorough characterization/screening of cell substrate starting material in order to identify which, if any, viral contaminants are present;

B. Assessment of risk by determination of the human tropism of the contaminants;

C. Establishment of an appropriate program of testing for adventitious viruses in unprocessed bulk;

D. Careful design of viral clearance studies using different methods of virus inactivation or removal in the same production process in order to achieve maximum viral clearance; and

E. Performance of studies which assess virus inactivation and removal.

GLOSSARY

Adventitious Virus: See Virus.

Cell Substrate: Cells used to manufacture product.

Endogenous Virus: See Virus.

Inactivation: Reduction of virus infectivity caused by chemical or physical modification.

In Vitro Cell Age: A measure of the period between thawing of the MCB vial(s) and harvest of the production vessel measured by elapsed chronological time in culture, population doubling level of the cells, or passage level of the cells when subcultivated by a defined procedure for dilution of the culture.
**Master Cell Bank (MCB):** An aliquot of a single pool of cells which generally has been prepared from the selected cell clone under defined conditions, dispensed into multiple containers, and stored under defined conditions. The MCB is used to derive all working cell banks. The testing performed on a new MCB (from a previous initial cell clone, MCB, or WCB) should be the same as for the original MCB, unless justified.

**Minimum Exposure Time:** The shortest period for which a treatment step will be maintained.

**Nonendogenous Virus:** See Virus.

**Process Characterization of Viral Clearance:** Viral clearance studies in which nonspecific “model” viruses are used to assess the robustness of the manufacturing process to remove and/or inactivate viruses.

**Process Evaluation Studies of Viral Clearance:** Viral clearance studies in which “relevant” and/or specific “model” viruses are used to determine the ability of the manufacturing process to remove and/or inactivate these viruses.

**Production Cells:** Cell substrate used to manufacture product.

**Unprocessed Bulk:** One or multiple pooled harvests of cells and culture media. When cells are not readily accessible, the unprocessed bulk would constitute fluid harvested from the fermenter.

**Virus:** Intracellularly replicating infectious agents that are potentially pathogenic, possess only a single type of nucleic acid (either ribonucleic acid (RNA) or DNA), are unable to grow and undergo binary fission, and multiply in the form of their genetic material.

**Adventitious Virus:** Unintentionally introduced contaminant virus.

**Endogenous Virus:** Viral entity whose genome is part of the germ line of the species of origin of the cell line and is covalently integrated into the genome of animal from which the parental cell line was derived. For the purposes of this document, intentionally introduced, nonintegrated viruses such as EBV used to immortalize cell substrates or Bovine Papilloma Virus fit in this category.

**Nonendogenous Virus:** Virus from external sources present in the MCB.

**Nonspecific Model Virus:** A virus used for characterization of viral clearance of the process when the purpose is to characterize the capacity of the manufacturing process to remove and/or inactivate viruses in general, i.e., to characterize the robustness of the purification process.

**Relevant Virus:** Virus used in process evaluation studies which is either the identified virus, or of the same species as the virus that is known, or likely to contaminate the cell substrate or any other reagents or materials used in the production process.

**Specific Model Virus:** Virus which is closely related to the known or suspected virus (same genus or family), having similar physical and chemical properties to those of the observed or suspected virus.

**Viral Clearance:** Elimination of target virus by removal of viral particles or inactivation of viral infectivity.

**Virus-like Particles:** Structures visible by electron microscopy which morphologically appear to be related to known viruses.

**Virus Removal:** Physical separation of virus particles from the intended product.

**Working Cell Bank (WCB):** The WCB is prepared from aliquots of a homogeneous suspension of cells obtained from culturing the MCB under defined culture conditions.

### APPENDICES

**Appendix 1: Products Derived from Characterized Cell Banks Which Were Subsequently Grown In Vivo**

For products manufactured from fluids harvested from animals inoculated with cells from characterized banks, additional information regarding the animals should be provided.

Whenever possible, animals used in the manufacture of biotechnological/biological products should be obtained from well defined, specific pathogen-free colonies. Adequate testing for appropriate viruses, such as those listed in Table 3, should be performed. Quarantine procedures for newly arrived as well as diseased animals should be described, and assurance provided that all containment, cleaning, and decontamination methodologies employed within the facility are adequate to contain the spread of adventitious agents. This may be accomplished through the use of a sentinel program. A listing of agents for which testing is performed should also be included. Veterinary support services should be available on-site or within easy access. The degree to which the vivarium is segregated from other areas of the manufacturing facility should be described. Personnel practices should be adequate to ensure safety.

Procedures for the maintenance of the animals should be fully described. These would include diet, cleaning and feeding schedules, provisions for periodic veterinary care if applicable, and details of special handling that the animals may require once inoculated. A description of the priming regimen(s) for the animals, the preparation of the inoculum, and the site and route of inoculation should also be included.

The primary harvest material from animals may be considered an equivalent stage of manufacture to unprocessed bulk harvest from a bioreactor. Therefore, all testing considerations previously outlined in section IV. of this document should apply. In addition, the manufacturer should assess the bioburden of the unprocessed bulk, determine whether the material is free of mycoplasma, and perform species-specific assay(s) as well as in vivo testing in adult and suckling mice.

**Appendix 2: The Choice of Viruses for Viral Clearance Studies**

A. **EXAMPLES OF USEFUL “Model” Viruses:**

1. Nonspecific “model” viruses representing a range of physico-chemical structures:
   - SV40 (Polyomavirus maccaca1), human polio virus 1 (Sabin), animal parvovirus or some other small, nonenveloped viruses;
• a parainfluenza virus or influenza virus, Sindbis virus or some other medium-to-large, enveloped, RNA viruses;
• a herpes virus (e.g., HSV-1 or a pseudorabies virus), or some other medium-to-large, DNA viruses.
These viruses are examples only and their use is not mandatory.
2. For rodent cell substrates murine retroviruses are commonly used as specific “model” viruses.

B. EXAMPLES OF VIRUSES THAT HAVE BEEN USED IN VIRAL CLEARANCE STUDIES

Several viruses that have been used in viral clearance studies are listed in Table A-1. However, since these are merely examples, the use of any of the viruses in the table is not considered mandatory and manufacturers are invited to consider other viruses, especially those that may be more appropriate for their individual production processes. Generally, the process should be assessed for its ability to clear at least three different viruses with differing characteristics.

Table A-1. Examples of Viruses Which Have Been Used in Viral Clearance Studies

<table>
<thead>
<tr>
<th>Virus</th>
<th>Family</th>
<th>Genus</th>
<th>Natural Host</th>
<th>Genome</th>
<th>Env</th>
<th>Size (nm)</th>
<th>Shape</th>
<th>Resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vesicular Stomatitis Virus</td>
<td>Paramyxovirus</td>
<td>Manivelovirus</td>
<td>Equine, bovine</td>
<td>RNA</td>
<td>yes</td>
<td>70 × 150</td>
<td>Bullet</td>
<td>Low</td>
</tr>
<tr>
<td>Parainfluenza Virus</td>
<td>Paramyxovirus</td>
<td>Paramyxovirus</td>
<td>Various</td>
<td>RNA</td>
<td>yes</td>
<td>100–200</td>
<td>Pleo/Spher</td>
<td>Low</td>
</tr>
<tr>
<td>BVDV</td>
<td>Retro</td>
<td>Type C oncovirus</td>
<td>Mouse</td>
<td>RNA</td>
<td>yes</td>
<td>80–110</td>
<td>Spherical</td>
<td>Low</td>
</tr>
<tr>
<td>Sindbis Virus</td>
<td>Toga</td>
<td>Alphavirus</td>
<td>Human</td>
<td>RNA</td>
<td>yes</td>
<td>60–70</td>
<td>Spherical</td>
<td>Low</td>
</tr>
<tr>
<td>BVDV</td>
<td>Flavi</td>
<td>Pestivirus</td>
<td>Bovine</td>
<td>RNA</td>
<td>yes</td>
<td>50–70</td>
<td>Pleo/Spher</td>
<td>Low</td>
</tr>
<tr>
<td>Pseudo-rabies virus</td>
<td>Herpes</td>
<td>DNA</td>
<td>Yes</td>
<td>60–120</td>
<td>200</td>
<td>Spherical</td>
<td>Med</td>
<td></td>
</tr>
<tr>
<td>Poliovirus Sabin Type 1</td>
<td>Picorna</td>
<td>Enterovirus</td>
<td>Human</td>
<td>RNA</td>
<td>no</td>
<td>25–30</td>
<td>Icosahedral</td>
<td>Med</td>
</tr>
<tr>
<td>Encephalomyocarditis Virus (EMCV)</td>
<td>Picorna</td>
<td>Cardiovirus</td>
<td>Mouse</td>
<td>RNA</td>
<td>no</td>
<td>25–30</td>
<td>Icosahedral</td>
<td>Med</td>
</tr>
<tr>
<td>Reovirus 3</td>
<td>Reo</td>
<td>Orthoreovirus</td>
<td>Various</td>
<td>RNA</td>
<td>no</td>
<td>60–80</td>
<td>Spherical</td>
<td>Med</td>
</tr>
<tr>
<td>SV 40</td>
<td>Papova</td>
<td>Polymavirus</td>
<td>Monkey</td>
<td>DNA</td>
<td>no</td>
<td>40–50</td>
<td>Icosahedral</td>
<td>Very high</td>
</tr>
<tr>
<td>Parvoviruses (canine, porcine)</td>
<td>Parvo</td>
<td>Parvovirus</td>
<td>Canine Porcine</td>
<td>DNA</td>
<td>no</td>
<td>18–24</td>
<td>Icosahedral</td>
<td>Very high</td>
</tr>
</tbody>
</table>

1 Resistance to physico-chemical treatments based on studies of production processes. Resistance is relative to the specific treatment and it is used in the context of the understanding of the biology of the virus and the nature of the manufacturing process. Actual results will vary according to the treatment. These viruses are examples only and their use is not considered mandatory.

Appendix 3: Statistical Considerations for Assessing Virus Assays

Virus titrations suffer the problems of variation common to all biological assay systems. Assessment of the accuracy of the virus titrations and reduction factors derived from them and the validity of the assays should be performed to define the reliability of a study. The objective of statistical evaluation is to establish that the study has been carried out to an acceptable level of virological competence.

1. Assay methods may be either quantal or quantitative. Quantal methods include infectivity assays in animals or in tissue-culture-infectious-dose (TCID) assays, in which the animal or cell culture is scored as either infected or not. Infectivity titers are then measured by the proportion of animals or culture infected. In quantitative methods, the infectivity measured varies continuously with the virus input. Quantitative methods include plaque assays where each plaque counted corresponds to a single infectious unit. Both quantal and quantitative assays are amenable to statistical evaluation.

2. Variation can arise within an assay as a result of dilution errors, statistical effects, and differences within the assay system which are either unknown or difficult to control. These effects are likely to be greater when different assay runs are compared (between-assay variation) than when results within a single assay run are compared (within-assay variation).

3. The 95 percent confidence limits for results of within-assay variation normally should be on the order of ±0.5 log10 of the mean. Within-assay variation can be assessed by standard textbook methods. Between-assay variation can be monitored by the inclusion of a reference preparation, the estimate of whose potency should be within approximately 0.5 log10 of the mean estimate established in the laboratory for the assay to be acceptable. Assays with lower precision may be acceptable with appropriate justification.

4. The 95 percent confidence limits for the reduction factor observed should be calculated wherever possible in studies of clearance of “relevant” and specific “model” viruses. If the 95 percent confidence limits for the viral assays of the starting material are +s, and for the viral assays of the material after the step are +a, the 95 percent confidence limits for the reduction factor are:

\[ \pm \sqrt{S^2 + a^2} \]

Appendix 4: Probability of Detection of Viruses at Low Concentrations

At low virus concentrations (e.g., in the range of 10 to 1,000 infectious particles per L) it is evident that a sample of a few milliliters may or may not contain infectious particles. The probability, p, that this sample does not contain infectious viruses is:
\[ p = \frac{(V-v)}{V} \]

where \( V \) (L) is the overall volume of the material to be tested; \( v \) (L) is the volume of the sample; and \( n \) is the absolute number of infectious particles statistically distributed in \( V \).

If \( V \gg v \), this equation can be approximated by the Poisson distribution:

\[ p = e^{-cv} \]

where \( c \) is the concentration of infectious particles per L.

or, \( c = \ln p/–v \)

As an example, if a sample volume of 1 mL is tested, the probabilities \( p \) at virus concentrations ranging from 10 to 1,000 infectious particles per L are:

<table>
<thead>
<tr>
<th>( c )</th>
<th>10</th>
<th>100</th>
<th>1,000</th>
</tr>
</thead>
<tbody>
<tr>
<td>( p )</td>
<td>0.99</td>
<td>0.90</td>
<td>0.37</td>
</tr>
</tbody>
</table>

This indicates that for a concentration of 1,000 viruses per L, in 37 percent of sampling, 1 mL will not contain a virus particle.

If only a portion of a sample is tested for virus and the test is negative, the amount of virus which would have to be present in the total sample in order to achieve a positive result should be calculated and this value taken into account when calculating a reduction factor. Confidence limits at 95 percent are desirable. However, in some instances, this may not be practical due to material limitations.

**Appendix 5: Calculation of Reduction Factors in Studies to Determine Viral Clearance**

The virus reduction factor of an individual purification or inactivation step is defined as the \( \log_{10} \) of the ratio of the virus load in the pre-purification material and the virus load in the post-purification material which is ready for use in the next step of the process. If the following abbreviations are used:

- Starting material: vol \( v' \); titer \( 10^{a'} \);
- virus load: \( (v')(10^{a'}) \);
- Final material: vol \( v'' \); titer \( 10^{a''} \);
- virus load: \( (v'')(10^{a''}) \);

the individual reduction factors \( R_i \) are calculated according to

\[ 10^{R_i} = \frac{(v')(10^{a'})}{(v'')(10^{a''})} \]

This formula takes into account both the titers and volumes of the materials before and after the purification step.

Because of the inherent imprecision of some virus titrations, an individual reduction factor used for the calculation of an overall reduction factor should be greater than 1.

The overall reduction factor for a complete production process is the sum logarithm of the reduction factors of the individual steps. It represents the logarithm of the ratio of the virus load at the beginning of the first process clearance step and at the end of the last process clearance step. Reduction factors are normally expressed on a logarithmic scale which implies that, while residual virus infectivity will never be reduced to zero, it may be greatly reduced mathematically.

**Appendix 6: Calculation of Estimated Particles per Dose**

This is applicable to those viruses for which an estimate of starting numbers can be made, such as endogenous retroviruses.

Example:

I. Assumptions

- Measured or estimated concentration of virus in cell culture harvest = \( 10^6 \)/mL
- Calculated viral clearance factor = \( >10^{15} \)
- Volume of culture harvest needed to make a dose of product = 1 L (\( 10^3 \) mL)

II. Calculation of Estimated Particles/Dose

\[ \frac{(10^7 \text{ virus units/mL}) \times (10^3 \text{ mL/dose})}{\text{Clearance factor} > 10^{15}} = 10^8 \text{ particles/dose} \]

\[ \frac{10^8 \text{ particles/dose}}{\text{Clearance factor} > 10^{15}} = <10^8 \text{ particles/dose} \]

Therefore, less than one particle per million doses would be expected.
(1053) CAPILLARY ELECTROPHORESIS

This chapter provides guidance and procedures used for characterization of biotechnology-derived articles by capillary electrophoresis. This chapter is harmonized with the corresponding chapter in JP and EP. Other characterization tests, also harmonized, are shown in Biotechnology-Derived Articles—Amino Acid Analysis (1052), Biotechnology-Derived Articles—Isoelectric Focusing (1054), Biotechnology-Derived Articles—Peptide Mapping (1055), Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis (1056), and Biotechnology-Derived Articles—Total Protein Assay (1057).

INTRODUCTION

Capillary electrophoresis is a physical method of analysis based on the migration, inside a capillary, of charged analytes dissolved in an electrolyte solution under the influence of a direct-current electric field. In this section we are describing four capillary electrophoresis methods: Capillary Zone Electrophoresis, Capillary Gel Electrophoresis, Capillary Isoelectric Focusing, and Micellar Electrokinetic Chromatography.

GENERAL PRINCIPLES

The migration velocity of the analyte under an electric field of intensity \( E \) is determined by the electrophoretic mobility of the analyte and the electroosmotic mobility of the buffer inside the capillary. The electrophoretic mobility of a solute (\( \mu_{ep} \)) depends on the characteristics of the solute (electrical charge, molecular size, and shape) and the characteristics of the buffer in which the migration takes place (type and ionic strength of the electrolyte, pH, viscosity, and additives). The electrophoretic velocity (\( v_{ep} \)) of a solute, assuming a spherical shape, is as follows:

\[
 v_{ep} = \mu_{ep} E = \left( \frac{q}{6\pi\eta r} \right) \left( \frac{V}{L} \right)
\]

in which \( q \) is the effective charge of the solute; \( \eta \) is the viscosity of the electrolyte solution; \( r \) is the Stoke’s radius of the solute; \( V \) is the applied voltage; and \( L \) is the total length of the capillary.

When an electric field is applied through the capillary filled with buffer, a flow of solvent, called electroosmotic flow, is generated inside the capillary. Its velocity depends on the electroosmotic mobility (\( \mu_{eo} \)), which in turn depends on the charge density on the capillary internal wall and the buffer characteristics. The electroosmotic velocity (\( v_{eo} \)) is given by the equation:

\[
 v_{eo} = \mu_{eo} E = \left( \frac{\varepsilon \zeta}{\eta} \right) \left( \frac{V}{L} \right)
\]

in which \( \varepsilon \) is the dielectric constant of the buffer; \( \zeta \) is the zeta potential of the capillary surface; and the other terms are as defined above.

The velocity of the solute (\( v \)) is given by the equation:

\[
 v = v_{ep} + v_{eo}
\]

The electrophoretic mobility of the analyte and the electroosmotic mobility may act in the same direction or in opposite directions, depending on the charge of the solute. In normal capillary electrophoresis, anions will migrate in the opposite direction to the electroosmotic flow and their velocities will be smaller than the electroosmotic velocity. Cations will migrate in the same direction as the electroosmotic flow and their velocities will be greater than the electroosmotic velocity. Under conditions in which there is a fast electroosmotic velocity with respect to the electrophoretic velocity of the solutes, both cations and anions can be separated in the same run. The time \( t \) taken by the solute to migrate the distance \( l \) from the injection end of the capillary to the detection point (capillary effective length) is as follows:

\[
 t = \frac{1}{v_{ep} + v_{eo}} = \frac{l(l)}{\mu_{ep} + \mu_{eo}}
\]

in which the other terms are as defined above.

In general, uncoated fused-silica capillaries above pH 3 have negative charge due to ionized silanol groups in the inner wall. Consequently, the electroosmotic flow is from anode to cathode. The electroosmotic flow must remain constant from run to run to obtain good reproducibility in the migration velocity of the solutes. For some applications, it might be necessary to reduce or suppress the electroosmotic flow by modifying the inner wall of the capillary or by changing the concentration, composition, and/or the pH of the buffer solution.

After the introduction of the sample into the capillary, each analyte ion of the sample migrates within the background electrolyte as an independent zone according to its electrophoretic mobility. Zone dispersion, that is the spreading of each solute band, results from different phenomena. Under ideal conditions, the sole contribution to the solute-zone broadening is molecular diffusion of the solute along the capillary (longitudinal diffusion). In this ideal case, the efficiency of the zone, expressed as the number of theoretical plates (\( N \)), is given by:
\[ N = \frac{(\mu_{ep} + \mu_{eo})Vl}{2DL} \]

in which \( D \) is the molecular diffusion coefficient of the solute in the buffer.

In practice, other phenomena, such as heat dissipation, sample adsorption onto the capillary wall, mismatched conductivity between sample and buffer, length of the injection plug, detector cell size, and unleveled buffer reservoirs, can also significantly contribute to band dispersion. Separation between two bands (expressed by the resolution, \( R_s \)) can be obtained by modification of the electrophoretic mobility of the analytes, by the electroosmotic mobility induced in the capillary, and by increasing the efficiency for the band of each analyte as follows:

\[ R_s = \frac{\sqrt{N}}{4(\mu_{ep} + \mu_{eo})} \]

in which \( \mu_{ep} \) and \( \mu_{epb} \) are the electrophoretic mobilities of the two analytes to be separated; \( \mu_{ep} \) is the average electrophoretic mobility of the two analytes calculated as:

\[ \mu_{ep} = \frac{1}{2}(\mu_{epb} + \mu_{epa}) \]

APPARATUS

An apparatus for capillary electrophoresis is composed of a high voltage controllable direct current power supply; two buffer reservoirs held at the same level and containing specified anodic and cathodic solutions; two electrode assemblies (cathode and anode) immersed in the buffer reservoirs and connected to the power supply; a separation capillary usually made of fused-silica, sometimes with an optical viewing window aligned with the detector; depending on the detector type, with the ends of the capillary placed in the buffer reservoirs and the capillary being filled with a solution specified in a given monograph; a suitable injection system; a detector capable of monitoring the amount of substance of interest passing through a segment of the separation capillary at a given time, generally based on absorption spectrophotometry (UV and visible), fluorimetry, conductimetric, amperometric, or mass spectrometric detection, depending on the specific applications, or even indirect detection to detect non-UV-absorbing and nonfluorescent compounds; a thermostatic system capable of maintaining a constant temperature inside the capillary, recommended to obtain good separation reproducibility; a recorder; and a suitable integrator or a computer.

The definition of the injection process and its automation are critical for precise quantitative analysis. Modes of injection include gravity, pressure or vacuum, or electrokinetic injection. The amount of each sample component introduced electrokinetically depends on its electrophoretic mobility, leading to possible discrimination using this injection mode.

It is expected that the capillary, the buffer solutions, the preconditioning method, the sample solution, and the migration conditions will be specified in the individual monograph. The electrolytic solution employed is filtered to remove particles and degassed to avoid bubble formation that could interfere with the detection system or interrupt the electrical contact in the capillary during the separation run. To achieve reproducible migration time of the solutes, it would be necessary to develop, for each analytical method, a rigorous rinsing routine.

Change to read:

CAPILLARY ZONE ELECTROPHORESIS

Principle

In capillary zone electrophoresis, analytes are separated in a capillary containing only buffer without any anticonvective medium. In this technique, separation takes place because the different components of the sample migrate as discrete bands with different velocities. The velocity of each band depends on the electrophoretic mobility of the solute and the electroosmotic flow on the capillary (see General Principles). Coated capillaries can be used to increase the separation capacity of those substances adsorbing on fused-silica surfaces.

This mode of capillary electrophoresis is appropriate for the analysis of small (MW < 2000) and large (2000 < MW < 100,000) molecules. Due to the high efficiency achieved in capillary zone electrophoresis, separation of molecules having only minute differences in their charge-to-mass ratio can be effected. This separation mode also allows the separation of chiral compounds by addition of chiral selectors to the separation buffer.

Optimization

Optimization of the separation is a complex process where several separation parameters can play a major role. The main factors to be considered in the development of the separations are instrumental and electrolytic solution parameters.
Instrumental Parameters

VOLTAGE

A Joule heating plot is useful in optimizing the applied voltage and column temperature. The separation time is inversely proportional to applied voltage. However, an increase in the voltage used can cause excessive heat production, giving rise to temperature and, as a result, viscosity gradients in the buffer inside the capillary, which causes band broadening and decreases resolution.

POLARITY

Electrode polarity can be normal (anode at the inlet and cathode at the outlet) and the electroosmotic flow will move toward the cathode. If the electrode polarity is reversed, the electroosmotic flow is away from the outlet and only charged analytes with electroosmotic mobilities greater than the electroosmotic flow will pass to the outlet.

TEMPERATURE

The main effect of temperature is observed on buffer viscosity and electrical conductivity, thus affecting migration velocity. In some cases, an increase in capillary temperature can cause a conformational change of some proteins, modifying their migration time and the efficiency of the separation.

CAPILLARY

The length and internal diameter of the capillary affects the analysis time, the efficiency of separations, and the load capacity. Increasing both effective length and total length can decrease the electric fields, at a constant voltage, which increases migration time. For a given buffer and electric field, heat dissipation (thus, sample band broadening) depends on the internal diameter of the capillary. The latter also affects the detection limit, depending on the sample volume injected into the capillary and the detection system used.

The adsorption of sample components on the capillary wall limits efficiency; therefore, methods to avoid these interactions should be considered in the development of a separation method. In the specific case of proteins, several strategies have been devised to avoid adsorption on the capillary wall. Some of these strategies (use of extreme pH and adsorption of positively charged buffer additives) only require modification of the buffer composition to prevent protein adsorption. Other strategies include the coating of the internal wall of the capillary with a polymer covalently bonded to the silica that prevents interaction between the proteins and the negatively charged silica surface. For this purpose, ready-to-use capillaries with coatings consisting of neutral-hydrophilic, cationic, and anionic polymers are commercially available.

Electrolytic Solution Parameters

BUFFER TYPE AND CONCENTRATIONS

Suitable buffers for capillary electrophoresis have an appropriate buffer capacity in the pH range of choice and low mobility to minimize current generation.

To minimize band distortion, it is important to match buffer–ion mobility to solute mobility whenever possible. The type of sample solvent used is important to achieve on-column sample focusing, which increases separation efficiency and improves detection. Also, an increase in buffer concentration at a given pH decreases electroosmotic flow and solute velocity.

BUFFER pH

The pH of the buffer can affect separation by modifying the charge of the analyte or additives and by changing the electroosmotic flow. For protein and peptide separation, a change in the pH of the buffer from above the isoelectric point to below the isoelectric point changes the net charge of the solute from negative to positive. An increase in the buffer pH generally increases the electroosmotic flow.

ORGANIC SOLVENTS

Organic modifiers, such as methanol, acetonitrile, and others, may be added to the aqueous buffer to increase the solubility of the solute or other additives and/or to affect the ionization degree of the sample components. The addition of these organic modifiers to the buffer generally causes a decrease in the electroosmotic flow.

ADDITIONS FOR CHIRAL SEPARATIONS

To separate chiral enantiomers, a chiral selector is added to the separation buffer. The most commonly used chiral selectors are cyclodextrins, although in some cases crown ethers, certain polysaccharides, or even proteins can be used. Because chiral recognition is governed by the different interactions between the chiral selector and each of the enantiomers, the resolution achieved for the chiral compounds depends largely on the type of chiral selector used. While developing a given separation it may be useful to test cyclodextrins having a different cavity size ($\alpha$, $\beta$, $\gamma$-cyclodextrin) or modified cyclodextrins with neutral (methyl, ethyl, hydroxalkyl, etc.) or ionizable (aminomethyl, carboxymethyl, sulfoalkylether, etc.) moieties. When using modified cyclodextrins, batch-to-batch variations in the degree of substitution of the cyclodextrins must be taken into account.
account because it will influence the selectivity. The resolution of chiral separations is also controlled by the concentration of the chiral selector, the composition and pH of the buffer, and the separation temperature. Organic additives, such as methanol or urea, can also affect the resolution of separation.

**CAPILLARY GEL ELECTROPHORESIS**

In capillary gel electrophoresis, separation takes place inside a capillary filled with a gel that acts as a molecular sieve. Molecules with similar charge-to-mass ratios are separated according to molecular size because smaller molecules move more freely through the network of the gel and therefore migrate faster than larger molecules. Different biological macromolecules (for example, proteins and DNA fragments), which often have similar charge-to-mass ratios, can thus be separated according to their molecular mass by capillary gel electrophoresis.

**Characteristics of Gels**

Two types of gels are used in capillary electrophoresis: permanently coated gels and dynamically coated gels. Permanently coated gels are prepared inside the capillary by polymerization of monomers. One example of such a gel is a cross-linked polyacrylamide. This type of gel is usually bonded to the fused-silica wall and cannot be removed without destroying the capillary. For protein analysis under reducing conditions, the separation buffer usually contains sodium dodecyl sulfate, and the sample is denatured by heating in a mixture of sodium dodecyl sulfate and 2-mercaptoethanol or dithiothreitol before injection. When nonreducing conditions are used (for example, analysis of an intact antibody), 2-mercaptoethanol and dithiothreitol are not used. Optimization of separation in a cross-linked gel is obtained by modifying the separation buffer (see *Capillary Zone Electrophoresis*) and by controlling the gel porosity during the gel preparation. For cross-linked polyacrylamide gels, the porosity can be modified by changing the concentration of acrylamide and/or the ratio of the cross-linker. As a rule, a decrease in the porosity of the gel leads to a decrease in the mobility of the solutes. Due to the rigidity of this type of gel, only electrokinetic injection can be used.

Dynamically coated gels are hydrophilic polymers (i.e., linear polyacrylamide, cellulose derivatives, dextran, etc.) which can be dissolved in aqueous separation buffers, giving rise to a separation medium that also acts as a molecular sieve. These polymeric separation media are easier to prepare than cross-linked polymers. They can be prepared in a vial and filled by pressure in a wall-coated capillary with no electroosmotic flow. Replacing the gel before every injection generally improves the separation reproducibility. The porosity of the dynamically coated gels can be increased by using polymers of higher molecular mass (at a given polymer concentration) or by decreasing the polymer concentration (for a given polymer molecular mass). A decrease in gel porosity leads to a decrease in the mobility of the solute for the same buffer. Both hydrodynamic and electrokinetic injection techniques can be used because the dissolution of these polymers in the buffer gives low viscosity solutions.

**CAPILLARY ISOELECTRIC FOCUSING**

**Principle**

In isoelectric focusing the molecules migrate under the influence of the electric field, so long as they are charged, in a pH gradient generated by ampholytes having pI values in a wide range (polymaminocarboxylic acids), dissolved in the separation buffer.

The three basic steps in capillary isoelectric focusing are loading, focusing, and mobilization.

**LOADING**

Two methods may be employed.

**Loading in One Step**—The sample is mixed with ampholytes and introduced into the capillary by pressure or vacuum.

**Sequential Loading**—A leading buffer, then the ampholytes, then the sample mixed with ampholytes, again ampholytes alone, and finally the terminating buffer are introduced into the capillary. The volume of the sample must be small enough so as not to modify the pH gradient.

**FOCUSING**

When the voltage is applied, ampholytes migrate toward the cathode or the anode according to their net charge, creating the pH gradient from anode (lower pH) to cathode (higher pH). During this step the components to be separated migrate until they reach a pH corresponding to their isoelectric point, and the current drops to very low values.

**MOBILIZATION**

If mobilization is required for detection, use one of the following methods. Three methods are available.

**Method 1**—Mobilization is accomplished during *Focusing*, under the influence of the electroosmotic flow when this flow is small enough to allow the focusing of the components.

**Method 2**—Mobilization is accomplished by application of positive pressure after *Focusing*.

**Method 3**—Mobilization is achieved after *Focusing*, by adding salts to the cathode reservoir or the anode reservoir, depending on the direction chosen for mobilization, in order to alter the pH in the capillary when the voltage is applied. As the pH is changed, the proteins and ampholytes are mobilized in the direction of the reservoir, which contains added salts, and pass the detector.
The separation achieved is expressed as $\Delta p_l$ and depends on the pH gradient ($dpH/dx$), the number of ampholytes having different pI values, the molecular diffusion coefficient ($D$), the intensity of the electric field ($E$), and the variation of the electrophoretic mobility of the analyte with the pH ($-d\mu/dpH$):

$$
\Delta p_l = 31 \sqrt{\frac{D(dpH/dx)}{E(-d\mu/dpH)}}
$$

**Optimization**

The major parameters that need to be considered in the development of separations are the following:

**VOLTAGE**

The use of high fields from 300 V/cm to 1,000 V/cm during Focusing.

**CAPILLARY**

Depending on the Mobilization strategy selected (see above), the electroosmotic flow must be reduced or suppressed. Coated capillaries tend to reduce the electroosmotic flow.

**SOLUTIONS**

The anode buffer reservoir is filled with a solution of a lower pH than the pI of the most acidic ampholyte, and the cathode reservoir is filled with a solution with a higher pH than the pI of the most basic ampholyte. Phosphoric acid for the anode and sodium hydroxide for the cathode are frequently used.

Addition of a polymer, like methylcellulose, in the ampholyte solution tends to suppress convective forces (if any) and electroosmotic flow by increasing the viscosity. Commercial ampholytes covering many pH ranges are available and may also be mixed to obtain an expanded pH range. Broad pH ranges are used to estimate the isoelectric point (pI), whereas narrower ranges are employed to improve accuracy. Calibration can be made by correlating migration time with the isoelectric point of a series of standard protein markers. During Focusing, precipitation of proteins at their isoelectric point can be prevented, if necessary, using buffer additives such as glycerol, surfactants, urea, or zwitterionic buffers. However, depending on the concentration, urea can denature proteins.

**MICELLAR ELECTROKINETIC CHROMATOGRAPHY (MEKC)**

**Principle**

Separation takes place in an electrolytic solution that contains a surfactant at a concentration above the critical micellar concentration (cmc). The solute molecules are distributed between the aqueous buffer and the pseudo-stationary phase composed by the micelles according to the solute's partition coefficient. The technique can be considered as a hybrid of electrophoresis and chromatography. It is a technique that can be used for the separation of both neutral and charged solutes maintaining the efficiency, speed, and instrumental suitability of capillary electrophoresis. One of the most widely used surfactants in MEKC is the anionic surfactant, sodium dodecyl sulfate, although other surfactants, such as cationic surfactant cetyl trimethyl ammonium salts, have also been used.

The separation mechanism is as follows. At neutral and alkaline pH, a strong electroosmotic flow is generated and moves the separation buffer ions in the direction of the cathode. If sodium dodecyl sulfate is used as surfactant, the electrophoretic migration of the anionic micelle is in the opposite direction, towards the anode. As a result, the overall micelle migration velocity is slowed compared to the bulk flow of the electrolytic solution. In the case of neutral solutes, because the anlyte can partition between the micelle and the aqueous buffer and has no electrophoretic mobility, the analyte migration velocity will depend only on the partition coefficient between the micelle and the aqueous buffer. In the electropherogram, the peaks corresponding to each uncharged solute are always between that of the electroosmotic flow marker and that of the micelle; and the time elapsed between these two peaks is called the separation window. For electrically charged solutes, the migration velocity depends on both the partition coefficient of the solute between the micelle and the aqueous buffer and on the electrophoretic mobility of the solute in the absence of micelles.

Because the mechanism in MEKC of neutral and weakly ionized solutes is essentially chromatographic, migration of the solute and resolution can be rationalized in terms of the retention factor of the solute ($k'$), also referred to as mass distribution ratio ($D_{mr}$), which is the ratio between the number of moles of solute in the micelle to those in the mobile phase. For a neutral compound, $k'$ is as follows:

$$k' = \frac{t_r - t_0}{t_0(1 - t_r/t_{mc})} = K \frac{V_s}{V_M}$$

in which $t_r$ is the migration time of the solute; $t_0$ is the analysis time of the unretained solute obtained by injecting an electroosmotic flow marker that does not enter the micelle (e.g., methanol); $t_{mc}$ is the micelle migration time measured by injecting a micelle marker, such as Sudan III, which migrates continuously associated in the micelle; $K$ is the partition coefficient of the solute; $V_s$ is the volume of the micellar phase; and $V_M$ is the volume of the mobile phase.
The resolution between two closely-migrating solutes \( (R_s) \) is as follows:

\[
R_s = \sqrt{\frac{N}{4}} \times \frac{\alpha - 1}{\alpha} \times \frac{k'_b}{k'_b + 1} \times \frac{1 - \left( \frac{t_0}{t_{mc}} \right)}{1 + k'_b \times \left( \frac{t_0}{t_{mc}} \right)}
\]

in which \( N \) is the number of theoretical plates for one of the solutes; \( \alpha \) is the selectivity; and \( k'_b \) and \( k'_a \) are retention factors for both solutes, respectively \((k'_b > k'_a)\).

Similar, but not identical, equations give \( k' \) and \( R_s \) values for electrically charged solutes.

**Optimization**

The main parameters to be considered in the development of separations by MEKC are instrumental and electrolytic solution parameters.

**INSTRUMENTAL PARAMETERS**

**Voltage**—Separation time is inversely proportional to applied voltage. However, an increase in voltage can cause excessive heat production that gives rise to temperature gradients and viscosity gradients of the buffer in the cross section of the capillary. This effect can be significant with high conductivity buffers, such as those containing micelles. Poor heat dissipation causes band broadening and decreases resolution.

**Temperature**—Variations in capillary temperature affect the partition coefficient of the solute between the buffer and the micelles, the critical micellar concentration, and the viscosity of the buffer. These parameters contribute to the migration time of the solute. The use of a good cooling system improves the reproducibility of the migration time for the solutes.

**Capillary**—As in Capillary Zone Electrophoresis, length and internal diameter of the capillary contribute to analysis time and efficiency of separations. Increasing both effective length and total length can decrease the electrical fields, working at constant voltage, and will increase migration time and improve the separation efficiency. The internal diameter controls heat dissipation, for a given buffer and electrical field, and consequently broadening of the sample band.

**ELECTROLYTIC SOLUTION PARAMETERS**

**Surfactant Type and Concentration**—The type of surfactant, as the stationary phase in chromatography, affects the resolution because it modifies separation selectively. The log \( k' \) of a neutral compound increases linearly with the concentration of surfactant in the mobile phase. When \( k' \) approaches the value of \( \sqrt{t_{mc}/t_0} \), resolution in MEKC reaches a maximum. Modifying the concentration of surfactant in the mobile phase changes the resolution.

**Buffer pH**—pH does not modify the partition coefficient of nonionized solutes, but it can modify the electroosmotic flow in uncoated capillaries. A decrease in the buffer pH decreases the electroosmotic flow and, therefore, increases the resolution of the neutral solutes in MEKC, resulting in a longer analysis time.

**Organic Solvents**—To improve MEKC separation of hydrophobic compounds, organic modifiers (methanol, propanol, acetonitrile, etc.) can be added to the electrolytic solution. The addition of these modifiers generally decreases migration time and selectivity of the separation. The addition of organic modifiers affects critical micellar concentration, thus, a given surfactant concentration can be used only with a certain percentage of organic modifier before the micellization is inhibited or adversely affected, resulting in the absence of micelles and, therefore, the absence of the partition. The dissociation of micelles in the presence of a high content of organic solvent does not always mean that the separation will no longer be possible, because in some cases, the hydrophobic interaction between the ionic surfactant monomer and the neutral solutes forms solvophobic complexes that can be separated electrophoretically.

**Additives for Chiral Separations**—For the separation of enantiomers using MEKC, a chiral selector is included in the micellar system, either covalently bound to the surfactant or added to the micellar separation electrolyte. Micelles that have a moiety with chiral discrimination properties include salts, N-dodecanoyl-l-amino acids, bile salts, etc. Chiral resolution can also be achieved using chiral discriminators, such as cyclodextrins, added to the electrolytic solutions that contain micellized achiral surfactants.

**Other Additives**—Selectivity can be modified by adding chemicals to the buffer. Addition of several types of cyclodextrins to the buffer is also used to reduce the interaction of hydrophobic solutes with the micelle, increasing the selectivity for this type of compound. The addition of substances able to modify solute-micelle interactions by adsorption on the latter has been used to improve the selectivity of the separations in MEKC. These additives may consist of a second surfactant (ionic or nonionic), which gives rise to mixed micelles or metallic cations that dissolve in the micelle and form coordination complexes with the solutes.

**Quantification**

Peak areas must be divided by the corresponding migration time to give the corrected area in order to compensate for the shift in migration time from run to run, thus reducing the variation of the response. Dividing the peak areas by migration time
will also compensate for the different responses of sample constituents with different migration times. Where an internal standard is used, check that no peak of the substance to be examined is masked by that of the internal standard.

**CALCULATIONS**

From the values obtained, calculate the content of a component or components being determined. When indicated, the percentage of one (or more) components of the sample to be examined is calculated by determining the corrected area(s) of the peak(s) as a percentage of the total of the corrected areas of all the peaks, excluding those due to solvents or any added reagents (normalization procedure). The use of an automatic integration system (integrator or data acquisition and processing system) is recommended.

**SYSTEM SUITABILITY**

In order to check the behavior of the capillary electrophoresis system, system suitability parameters are used. The choice of these parameters depends on the mode of capillary electrophoresis used. The parameters include the following: retention factor \( k' \) used only for Micellar Electrokinetic Chromatography, apparent number of theoretical plates \( (N) \), the symmetry factor \( (A_s) \), and the resolution \( (R_s) \). Note that in previous sections, the theoretical expressions for \( N \) and \( R_s \) have been described, but more practical equations that allow for the determination of these suitability parameters using the electropherograms are described below.

**Apparent Number of Theoretical Plates**

The apparent number of theoretical plates \( (N) \) may be calculated from the formula:

\[
N = 5.54 \left( \frac{t_R}{w_{1/2}} \right)^2
\]

in which \( t_R \) is the migration time or distance along the baseline between the point of injection and the perpendicular dropped from the maximum of the peak corresponding to the component; and \( w_{1/2} \) is the peak width at half-height.

**Resolution**

The resolution \( (R_s) \) between peaks of similar heights of two components may be calculated from the formula:

\[
R_s = 1.18 \left( \frac{t_{R2} - t_{R1}}{w_{h1} + w_{h2}} \right)
\]

in which \( t_{R1} \) and \( t_{R2} \) are the migration times or distances along the baseline between the point of injection and the perpendiculars dropped from the maxima of two adjacent peaks; and \( w_{h1} \) and \( w_{h2} \) are the peak widths at half-height.

When appropriate, the resolution \( (R_s) \) may also be calculated by measuring the height of the valley \( (H_v) \) between two partly resolved peaks in a standard preparation, the height of the smaller peak \( (H_p) \), and calculating the peak-to-valley ratio:

\[
p/v = H_p/H_v
\]

**Symmetry Factor**

The symmetry factor of a peak \( (A_s) \) may be calculated using the formula:

\[
A_s = \frac{w_0.05}{2d}
\]

in which \( w_{0.05} \) is the width of the peak at one-twentieth of the peak height; and \( d \) is the distance between the perpendicular dropped from the peak maximum and the leading edge of the peak at one-twentieth of the peak height.

Other suitability parameters include tests for area repeatability (standard deviation of areas or of area/migration time) and tests for migration time repeatability (standard deviation of migration time). Migration time repeatability provides a test for the suitability of the capillary washing procedures. An alternative practice to avoid the lack of repeatability of the migration time is to use a migration time relative to an internal standard.

**Signal-to-Noise Ratio**

A test for the verification of the signal-to-noise ratio for a standard preparation or the determination of the limit of quantification may also be useful for the determination of related substances. The detection limit and quantification limit correspond to a signal-to-noise ratio of 3 and 10, respectively. The signal-to-noise ratio \( (S/N) \) is calculated as follows:

\[
S/N = 2H/h
\]

in which \( H \) is the height of the peak corresponding to the component concerned in the electropherogram obtained with the specified reference solution, measured from the maximum of the peak to the extrapolated baseline of the signal observed over a
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distance equal to twenty times the width at half-height; and $h$ is the range of the background in an electropherogram obtained after injection of a blank, observed over a distance equal to twenty times the width at the half-height of the peak in the electropherogram obtained with the prescribed reference solution and, if possible, situated equally around the place where this peak would be found.

\section*{(1056) BIOTECHNOLOGY-DERIVED ARTICLES—POLYACRYLAMIDE GEL ELECTROPHORESIS}

\subsection*{INTRODUCTION}

\subsection*{Scope}

Polyacrylamide gel electrophoresis is used for the qualitative characterization of proteins in biological preparations, for control of purity, and for quantitative determinations.

\subsection*{Purpose}

Analytical gel electrophoresis is an appropriate method with which to identify and to assess the homogeneity of proteins in pharmaceutical preparations. The method is routinely used for the estimation of protein subunit molecular masses and for determination of the subunit compositions of purified proteins. Ready-to-use gels and reagents are commercially available and can be used instead of those described in this text, provided that they give equivalent results and that they meet the validity requirements given in \textit{Validation of the Test} (below).

\section*{CHARACTERISTICS OF POLYACRYLAMIDE GELS}

The sieving properties of polyacrylamide gels are established by the three-dimensional network of fibers and pores that is formed as the bifunctional bisacrylamide cross-links adjacent polyacrylamide chains. Polymerization usually is catalyzed by a free radical–generating system composed of ammonium persulfate and $N,N',N''$-tetramethylethylenediamine (TEMED).

As the acrylamide concentration of a gel increases, its effective pore size decreases. The effective pore size of a gel is operationally defined by its sieving properties, that is, by the resistance it imparts to the migration of macromolecules. There are limits on the acrylamide concentrations that can be used. At high acrylamide concentrations, gels break much more easily and are difficult to handle. As the pore size of a gel decreases, the migration rate of a protein through the gel decreases. By adjusting the pore size of a gel through manipulating the acrylamide concentration, analysts can optimize the resolution of the method for a given protein product. Thus, a given gel is physically characterized by its respective composition of acrylamide and bisacrylamide.

In addition to the composition of the gel, the state of the protein is an important component of electrophoretic mobility. In the case of proteins, the electrophoretic mobility depends on the $pK$ value of the charged groups and the size of the molecule. It is influenced by the type, the concentration, and the pH of the buffer, by the temperature and the field strength; and by the nature of the support material.

\section*{DENATURING POLYACRYLAMIDE GEL ELECTROPHORESIS}

The method cited as an example is limited to the analysis of monomeric polypeptides with a mass range of 14,000–100,000 Da. It is possible to extend this mass range by various techniques (e.g., gradient gels and particular buffer system). For instance, tricine–sodium dodecyl sulfate (SDS) gels, using tricine instead of glycine (in the method described here) as the trailing ion in the electrophoresis running buffer, can separate very small proteins and peptides under 10,000–15,000 Da.

Denaturing polyacrylamide gel electrophoresis using glycine SDS (SDS-PAGE) is the most common mode of electrophoresis used in assessing the pharmaceutical quality of protein products and is the focus of the example method. Typically, analytical electrophoresis of proteins is carried out in polyacrylamide gels under conditions that ensure dissociation of the proteins into their individual polypeptide subunits and that minimize aggregation. Most commonly, the strongly anionic detergent SDS is used in combination with heat to dissociate the proteins before they are loaded on the gel. The denatured polypeptides bind to SDS, become negatively charged, and exhibit a consistent charge-to-mass ratio regardless of protein type. Because the amount of SDS bound is almost always proportional to the molecular mass of the polypeptide and is independent of its sequence, SDS–polypeptide complexes migrate through polyacrylamide gels with mobilities dependent on the size of the polypeptide.

The electrophoretic mobilities of the resultant detergent–polypeptide complexes all assume the same functional relationship to their molecular masses. SDS complexes migrate toward the anode in a predictable manner; low-molecular-mass complexes migrate faster than larger ones. The molecular mass of a protein therefore can be estimated from its relative mobility in calibrated SDS-PAGE, and the intensity of a single band relative to other undesired bands in such a gel can be a measure of purity.

Modifications to the polypeptide backbone, such as N- or O-linked glycosylation, can change the apparent molecular mass of a protein, because SDS does not bind to a carbohydrate moiety in a manner similar to that of a polypeptide; therefore, a consistent charge-to-mass ratio is not maintained.
Depending on the extent of glycosylation and other posttranslational modifications, the apparent molecular mass of proteins may not be a true reflection of the mass of the polypeptide chain.

Reducing Conditions

Polypeptide subunits and three-dimensional structure often are maintained in proteins by the presence of disulfide bonds. A goal of SDS-PAGE analysis under reducing conditions is to disrupt this structure by reducing disulfide bonds. Complete denaturation and dissociation of proteins by treatment with 2-mercaptoethanol (2-ME) or dithiothreitol (DTT) results in unfolding of the polypeptide backbone and subsequent complexation with SDS. Using these conditions, analysts can reasonably calculate the molecular mass of the polypeptide by linear regression (or, more closely, by nonlinear regression) in the presence of suitable molecular mass standards.

Nonreducing Conditions

For some analyses, complete dissociation of the protein into subunit peptides is not desirable. In the absence of treatment with reducing agents such as 2-ME or DTT, disulfide covalent bonds remain intact, preserving the oligomeric form of the protein. Oligomeric SDS–protein complexes migrate more slowly than their SDS–polypeptide subunits. In addition, nonreduced proteins may not be completely saturated with SDS and hence may not bind the detergent in a constant mass ratio. Moreover, intrachain disulfide bonds constrain the molecular shape, usually in such a way that reduces the Stokes radius of the molecule, thereby reducing the apparent molecular mass, M. This makes molecular mass determinations of these molecules by SDS-PAGE less straightforward than analyses of fully denatured polypeptides because it is necessary that both standards and unknown proteins be in similar configurations for valid comparisons.

CHARACTERISTICS OF DISCONTINUOUS BUFFER SYSTEM GEL ELECTROPHORESIS

The most popular electrophoretic method for the characterization of complex mixtures of proteins uses a discontinuous buffer system involving two contiguous but distinct gels: a resolving or separating (lower) gel and a stacking (upper) gel. The two gels are cast with different porosities, pH, and ionic strengths. In addition, different mobile ions are used in the gel and electrode buffers. The buffer discontinuity acts to concentrate large-volume samples in the stacking gel, resulting in improved resolution. When power is applied, a voltage drop develops across the sample solution and drives the proteins into the stacking gel. Glycinate ions from the electrode buffer follow the proteins into the stacking gel. A moving boundary region is rapidly formed, with the highly mobile chloride ions in the front and the relatively slow glycinate ions in the rear. A localized high-voltage gradient forms between the leading and trailing ion fronts, causing the SDS–protein complexes to form into a thin zone (stack) and to migrate between the chloride and glycinate phases. Within broad limits, regardless of the height of the applied sample, all SDS–proteins condense into a very narrow region and enter the resolving gel as a well-defined, thin zone of high protein density. The large-pore stacking gel does not retard the migration of most proteins and serves mainly as an anticonvective medium. At the interface of the stacking and resolving gels, the proteins undergo a sharp increase in retardation due to the restrictive pore size of the resolving gel and the buffer discontinuity, which also contributes to focusing of the proteins. Once in the resolving gel, proteins continue to be slowed by the sieving of the matrix. The glycinate ions overtake the proteins, which then move in a space of uniform pH formed by the tris(hydroxymethyl)aminomethane (Tris) and glycine. Molecular sieving causes the SDS–polypeptide complexes to separate on the basis of their molecular masses.

PREPARING VERTICAL DISCONTINUOUS BUFFER SDS POLYACRYLAMIDE GELS

This section describes the preparation of gels using particular instrumentation. This does not apply to precast gels. For precast gels or any other commercially available equipment, the manufacturer’s instructions must be used for guidance.

The use of commercial reagents that have been purified in solution is recommended. When this is not the case and when the purity of the reagents used is not sufficient, a pretreatment is applied. For instance, any solution sufficiently impure to require filtration must also be deionized with a mixed-bed (anion–cation exchange) resin to remove acrylic acid and other charged degradation products. When stored according to recommendations, acrylamide/bisacrylamide solutions and solid persulfate are stable for long periods.

Gel Stock Solutions

30% ACRYLAMIDE–BISACRYLAMIDE SOLUTION

Prepare a solution containing 290 g of acrylamide and 10 g of methylenebisacrylamide per liter of water. Filter.

AMMONIUM PERSULFATE SOLUTION

Prepare a small quantity of solution having a concentration of 100 g/L of ammonium persulfate. [Note—Ammonium persulfate provides the free radicals that drive polymerization of acrylamide and bisacrylamide. Because ammonium persulfate decomposes rapidly, fresh solutions must be prepared daily.]
TEMED

Use an electrophoresis-grade reagent.

SDS SOLUTION

This is a 100 g/L solution of electrophoresis-grade SDS.

1.5 M BUFFER SOLUTION

Dissolve 90.8 g of Tris in 400 mL of water. Adjust the pH to 8.8 with hydrochloric acid, and dilute to 500.0 mL with water.

1 M BUFFER SOLUTION

Dissolve 60.6 g of Tris in 400 mL of water. Adjust the pH to 6.8 with hydrochloric acid, and dilute to 500.0 mL with water.

Assembling the Gel-Molding Cassette

Clean the two glass plates (size, e.g., 10 cm × 8 cm), the polytetrafluoroethylene comb, the two spacers, and the silicone rubber tubing (e.g., 0.6 mm diameter × 35 cm length) with mild detergent; rinse extensively with water, followed by dehydrated alcohol; and allow the plates to dry at room temperature. Lubricate the spacers and the tubing with nonsilicone grease. Apply the spacers along each of the two short sides of the glass plate 2 mm away from the edges and 2 mm away from the long side corresponding to the bottom of the gel. Begin to lay the tubing on the glass plate by using one spacer as a guide. Carefully twist the tubing at the bottom of the spacer, and follow the long side of the glass plate. While holding the tubing with one finger along the long side, twist the tubing again and lay it on the second short side of the glass plate, using the spacer as a guide. Place the second glass plate in perfect alignment, and hold the mold together by hand pressure.

Apply two clamps on each of the two short sides of the mold. Carefully apply four clamps on the longer side of the gel mold, thus forming the bottom of the gel mold. Verify that the tubing is running along the edge of the glass plates and has not been extruded while the clamps were placed. The gel mold is now ready for pouring the gel.

Preparation of the Gel

In a discontinuous buffer SDS polyacrylamide gel, it is recommended to pour the resolving gel, let the gel set, and then pour the stacking gel, because the composition of the two gels in acrylamide-bisacrylamide, buffer, and pH are different.

PREPARATION OF THE RESOLVING GEL

In a conical flask, prepare the appropriate volume of solution containing the desired concentration of acrylamide for the resolving gel, using the values given in Table 1. Mix the components in the order shown. Where appropriate, before adding the Ammonium Persulfate Solution and the TEMED, filter the solution if necessary under vacuum through a cellulose acetate membrane (pore diameter: 0.45 µm). Keep the solution under vacuum, while swirling the filtration unit, until no more bubbles are formed in the solution. Add appropriate amounts of Ammonium Persulfate Solution and TEMED, as indicated in Table 1, swirl, and pour immediately into the gap between the two glass plates of the mold. Leave sufficient space for the stacking gel (the length of the teeth of the comb plus 1 cm). Using a tapered glass pipet, carefully overlay the solution with water-saturated isobutanol. Leave the gel in a vertical position at room temperature to allow polymerization.

<table>
<thead>
<tr>
<th>Table 1. Preparation of the Resolving Gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Component Volume (mL) per Gel Mold Volume Below</td>
</tr>
<tr>
<td>Solution component</td>
</tr>
<tr>
<td>---------------------</td>
</tr>
<tr>
<td>6% Acrylamide</td>
</tr>
<tr>
<td>Water</td>
</tr>
<tr>
<td>30% Acrylamide–Bisacrylamide Solution</td>
</tr>
<tr>
<td>1.5 M Buffer Solution</td>
</tr>
<tr>
<td>SDS Solution</td>
</tr>
<tr>
<td>Ammonium Persulfate Solution</td>
</tr>
<tr>
<td>TEMED</td>
</tr>
<tr>
<td>8% Acrylamide</td>
</tr>
<tr>
<td>Water</td>
</tr>
<tr>
<td>30% Acrylamide–Bisacrylamide Solution</td>
</tr>
</tbody>
</table>
### Table 1. Preparation of the Resolving Gel (continued)

<table>
<thead>
<tr>
<th>Solution component</th>
<th>5 mL</th>
<th>10 mL</th>
<th>15 mL</th>
<th>20 mL</th>
<th>25 mL</th>
<th>30 mL</th>
<th>40 mL</th>
<th>50 mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5 M Buffer Solution</td>
<td>1.3</td>
<td>2.5</td>
<td>3.8</td>
<td>5.0</td>
<td>6.3</td>
<td>7.5</td>
<td>10.0</td>
<td>12.5</td>
</tr>
<tr>
<td>SDS Solution</td>
<td>0.05</td>
<td>0.1</td>
<td>0.15</td>
<td>0.2</td>
<td>0.25</td>
<td>0.3</td>
<td>0.4</td>
<td>0.5</td>
</tr>
<tr>
<td>Ammonium Persulfate Solution</td>
<td>0.05</td>
<td>0.1</td>
<td>0.15</td>
<td>0.2</td>
<td>0.25</td>
<td>0.3</td>
<td>0.4</td>
<td>0.5</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.003</td>
<td>0.006</td>
<td>0.009</td>
<td>0.012</td>
<td>0.015</td>
<td>0.018</td>
<td>0.024</td>
<td>0.03</td>
</tr>
</tbody>
</table>

10% Acrylamide

| Water         | 1.9 | 4.0 | 5.9 | 7.9 | 9.9 | 11.9 | 15.9 | 19.8 |

30% Acrylamide–Bisacrylamide Solution

| 1.7 | 3.3 | 5.0 | 6.7 | 8.3 | 10.0 | 13.3 | 16.7 |

1.5 M Buffer Solution

| 1.3 | 2.5 | 3.8 | 5.0 | 6.3 | 7.5 | 10.0 | 12.5 |

SDS Solution

| 0.05 | 0.1 | 0.15 | 0.2 | 0.25 | 0.3 | 0.4 | 0.5 |

Ammonium Persulfate Solution

| 0.05 | 0.1 | 0.15 | 0.2 | 0.25 | 0.3 | 0.4 | 0.5 |

TEMED

| 0.002 | 0.004 | 0.006 | 0.008 | 0.01 | 0.012 | 0.016 | 0.02 |

12% Acrylamide

| Water | 1.6 | 3.3 | 4.9 | 6.6 | 8.2 | 9.9 | 13.2 | 16.5 |

30% Acrylamide–Bisacrylamide Solution

| 2.0 | 4.0 | 6.0 | 8.0 | 10.0 | 12.0 | 16.0 | 20.0 |

1.5 M Buffer Solution

| 1.3 | 2.5 | 3.8 | 5.0 | 6.3 | 7.5 | 10.0 | 12.5 |

SDS Solution

| 0.05 | 0.1 | 0.15 | 0.2 | 0.25 | 0.3 | 0.4 | 0.5 |

Ammonium Persulfate Solution

| 0.05 | 0.1 | 0.15 | 0.2 | 0.25 | 0.3 | 0.4 | 0.5 |

TEMED

| 0.002 | 0.004 | 0.006 | 0.008 | 0.01 | 0.012 | 0.016 | 0.02 |

14% Acrylamide

| Water | 1.4 | 2.7 | 3.9 | 5.3 | 6.6 | 8.0 | 10.6 | 13.8 |

30% Acrylamide–Bisacrylamide Solution

| 2.3 | 4.6 | 7.0 | 9.3 | 11.6 | 13.9 | 18.6 | 23.2 |

1.5 M Buffer Solution

| 1.2 | 2.5 | 3.6 | 5.0 | 6.3 | 7.5 | 10.0 | 12.5 |

SDS Solution

| 0.05 | 0.1 | 0.15 | 0.2 | 0.25 | 0.3 | 0.4 | 0.5 |

Ammonium Persulfate Solution

| 0.05 | 0.1 | 0.15 | 0.2 | 0.25 | 0.3 | 0.4 | 0.5 |

TEMED

| 0.002 | 0.004 | 0.006 | 0.008 | 0.01 | 0.012 | 0.016 | 0.02 |

15% Acrylamide

| Water | 1.1 | 2.3 | 3.4 | 4.6 | 5.7 | 6.9 | 9.2 | 11.5 |

30% Acrylamide–Bisacrylamide Solution

| 2.5 | 5.0 | 7.5 | 10.0 | 12.5 | 15.0 | 20.0 | 25.0 |

1.5 M Buffer Solution

| 1.3 | 2.5 | 3.8 | 5.0 | 6.3 | 7.5 | 10.0 | 12.5 |

SDS Solution

| 0.05 | 0.1 | 0.15 | 0.2 | 0.25 | 0.3 | 0.4 | 0.5 |

Ammonium Persulfate Solution

| 0.05 | 0.1 | 0.15 | 0.2 | 0.25 | 0.3 | 0.4 | 0.5 |

TEMED

| 0.002 | 0.004 | 0.006 | 0.008 | 0.01 | 0.012 | 0.016 | 0.02 |

**PREPARATION OF THE STACKING GEL**

After polymerization is complete (about 30 min), pour off the isobutanol, and wash the top of the gel several times with water to remove the isobutanol overlay and any unpolymerized acrylamide. Drain as much fluid as possible from the top of the gel, and then remove any remaining water with the edge of a paper towel.

In a conical flask, prepare the appropriate volume of solution containing the desired concentration of acrylamide, using the values given in Table 2. Mix the components in the order shown. Where appropriate, before adding the Ammonium Persulfate Solution and the TEMED, filter the solution if necessary under vacuum through a cellulose acetate membrane (pore diameter:
Keep the solution under vacuum, while swirling the filtration unit, until no more bubbles are formed in the solution. Add appropriate amounts of *Ammonium Persulfate Solution* and *TEMED*, as indicated in Table 2. Swirl, and pour immediately into the gap between the two glass plates of the mold directly onto the surface of the polymerized resolving gel. Immediately insert a clean polytetrafluoroethylene comb into the stacking gel solution, being careful to avoid trapping air bubbles. Add more stacking gel solution to fill the spaces of the comb completely. Leave the gel in a vertical position, and allow it to polymerize at room temperature.

### Table 2. Preparation of the Stacking Gel

<table>
<thead>
<tr>
<th>Component Volume (mL) per Gel Mold Volume Below</th>
<th>1 mL</th>
<th>2 mL</th>
<th>3 mL</th>
<th>4 mL</th>
<th>5 mL</th>
<th>6 mL</th>
<th>8 mL</th>
<th>10 mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>0.68</td>
<td>1.4</td>
<td>2.1</td>
<td>2.7</td>
<td>3.4</td>
<td>4.1</td>
<td>5.5</td>
<td>6.8</td>
</tr>
<tr>
<td>30% Acrylamide–Bisacrylamide Solution</td>
<td>0.17</td>
<td>0.33</td>
<td>0.5</td>
<td>0.67</td>
<td>0.83</td>
<td>1.0</td>
<td>1.3</td>
<td>1.7</td>
</tr>
<tr>
<td>1.0 M Buffer Solution</td>
<td>0.13</td>
<td>0.25</td>
<td>0.38</td>
<td>0.5</td>
<td>0.63</td>
<td>0.75</td>
<td>1.0</td>
<td>1.25</td>
</tr>
<tr>
<td>SDS Solution</td>
<td>0.01</td>
<td>0.02</td>
<td>0.03</td>
<td>0.04</td>
<td>0.05</td>
<td>0.06</td>
<td>0.08</td>
<td>0.1</td>
</tr>
<tr>
<td>Ammonium Persulfate Solution</td>
<td>0.01</td>
<td>0.02</td>
<td>0.03</td>
<td>0.04</td>
<td>0.05</td>
<td>0.06</td>
<td>0.08</td>
<td>0.1</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.001</td>
<td>0.002</td>
<td>0.003</td>
<td>0.004</td>
<td>0.005</td>
<td>0.006</td>
<td>0.008</td>
<td>0.01</td>
</tr>
</tbody>
</table>

### Preparation of the Sample

Unless otherwise specified in the specific monograph, the samples can be prepared as follows:

**SDS-PAGE SAMPLE BUFFER (CONCENTRATED)**

Dissolve 1.89 g of Tris, 5.0 g of sodium lauryl sulfate, and 50 mg of bromophenol blue in water. Add 25.0 mL of glycerol, and dilute to 100 mL with water. Adjust the pH to 6.8 with hydrochloric acid, and dilute to 125 mL with water.

**SDS-PAGE SAMPLE BUFFER FOR REDUCING CONDITIONS (CONCENTRATED)**

Dissolve 3.78 g of Tris, 10.0 g of SDS, and 100 mg of bromophenol blue in water. Add 50.0 mL of glycerol, and dilute to 200 mL with water. Add 25.0 mL of 2-ME. Adjust to pH 6.8 with hydrochloric acid, and dilute to 250.0 mL with water. Alternatively, DTT can be used as reducing agent instead of 2-ME. In this case prepare the sample buffer as follows: Dissolve 3.78 g of Tris, 10.0 g of SDS, and 100 mg of bromophenol blue in water. Add 50.0 mL of glycerol, and dilute to 200 mL with water. Adjust to pH 6.8 with hydrochloric acid, and dilute to 250.0 mL with water. Immediately before use, add DTT to a final concentration of 100 mM.

**SDS-PAGE RUNNING BUFFER**

Dissolve 151.4 g of Tris, 721.0 g of glycine, and 50.0 g of sodium lauryl sulfate in water, and dilute to 5000 mL with the same solvent. Immediately before use, dilute to 10 times its volume with water, and mix. Measure the pH of the diluted solution. The pH is between 8.1 and 8.8.

**SAMPLE SOLUTION (NONREDUCING CONDITIONS)**

Mix equal volumes of: a mixture comprising water plus the preparation or the reference solutions, and *SDS-PAGE Sample Buffer (Concentrated)*.

**SAMPLE SOLUTION (REDUCING CONDITIONS)**

Mix equal volumes of: a mixture comprising water plus the preparation or the reference solutions, and *SDS-PAGE Sample Buffer for Reducing Conditions (Concentrated)* containing 2-ME (or DTT) as the reducing agent. The concentration prescribed in the monograph can vary depending on the protein and staining method.

Sample treatment: Keep for 5 min in a boiling water bath or in a block heater set at 100°, and then chill. (Note that temperature and time may vary in the monograph because protein cleavage may occur during the heat treatment.)

### Mounting the Gel in the Electrophoresis Apparatus and Electrophoretic Separation

After polymerization is complete (about 30 min), remove the polytetrafluoroethylene comb carefully. Rinse the wells immediately with water or with the *SDS-PAGE Running Buffer* to remove any unpolymerized acrylamide. If necessary, straighten the teeth of the stacking gel with a blunt hypodermic needle attached to a syringe. Remove the clamps on one short side, carefully pull out the tubing, and replace the clamps. Proceed similarly on the other short side. Remove the tubing from the bottom part of the gel. Mount the gel in the electrophoresis apparatus. Add the electrophoresis buffers to the top and bottom reservoirs. Remove any bubbles that become trapped at the bottom of the gel between the glass plates. This is best done with a bent hypodermic needle attached to a syringe. Never prerun the gel before loading the samples, because this will destroy the discontinuity of the buffer systems. Before loading the sample, carefully rinse each well with *SDS-PAGE Running Buffer*. Prepare
the test and reference solutions in the recommended sample buffer, and treat as specified in the individual monograph. Apply the appropriate volume of each solution to the stacking gel wells. Start the electrophoresis using the conditions recommended by the equipment manufacturer. Manufacturers of SDS-PAGE equipment may provide gels of different surface area and thickness, and electrophoresis running time and current or voltage may vary in order to achieve optimal separation. Check that the dye front is moving into the resolving gel. When the dye is near the bottom of the gel stop the electrophoresis. Remove the gel assembly from the apparatus, and carefully separate the glass plates. Remove the spacers, cut off and discard the stacking gel, and immediately proceed with staining.

**SODIUM DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS—GRADIENT CONCENTRATION GELS**

Gradient gels (resolving gels) are prepared with an increasing concentration of acrylamide from the top to the bottom. Preparation of gradient gels requires a gradient-forming apparatus. Ready-to-use gradient gels are commercially available with specific recommended protocols. Gradient gels offer some advantages over fixed-concentration gels. Some proteins that co-migrate on fixed-concentration gels can be resolved within gradient gels. During electrophoresis the proteins migrate until the pore size stops further progress, and therefore a stacking effect occurs, resulting in sharper bands. According to Table 3, gradient gels also allow separation of a wider range of protein molecular masses than do single, fixed-concentration gels.

Table 3 gives suggested compositions of the linear gradient, relating the range of acrylamide concentrations to the appropriate protein molecular ranges. Note that other gradient shapes (e.g., concave) can be prepared for specific applications.

<table>
<thead>
<tr>
<th>Acrylamide (%)</th>
<th>Protein Range (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5–15</td>
<td>20–250</td>
</tr>
<tr>
<td>5–20</td>
<td>10–200</td>
</tr>
<tr>
<td>10–20</td>
<td>10–150</td>
</tr>
<tr>
<td>8–20</td>
<td>8–150</td>
</tr>
</tbody>
</table>

Gradient gels also are used for molecular mass determination and protein purity determination.

**DETECTION OF PROTEINS IN GELS**

Coomassie and silver staining are the most common protein staining methods and are described in more detail below. Several other commercial stains, detection methods, and commercial kits are available. For example, fluorescent stains are visualized using a fluorescent imager and often provide a linear response over a wide range of protein concentrations—often several orders of magnitude, depending on the protein.

Coomassie staining has a protein detection level of approximately 1–10 µg of protein per band. Silver staining is the most sensitive method for staining proteins in gels, and a band containing 10–100 ng can be detected. These figures are considered robust in the context of these gels. Improved sensitivity of one or two orders of magnitude has been reported in the literature.

Coomassie staining responds in a more linear manner than silver staining, but the response and range depend on the protein and development time. Both Coomassie and silver staining can be less reproducible if staining is stopped in a subjective manner, i.e., when the analyst deems the staining satisfactory. Wide dynamic ranges of reference proteins are important to use because they help assess the intra-experimental sensitivity and linearity. All gel-staining steps are done while wearing gloves, at room temperature, with gentle shaking (e.g., on an orbital shaker platform), and using any convenient container.

**Staining Reagents**

**DESTAINING SOLUTION**

Prepare a mixture of 1 volume of glacial acetic acid, 4 volumes of methanol, and 5 volumes of water.

**COOMASSIE STAINING SOLUTION**

Prepare a 1.25 g/L solution of acid blue 83 in Destaining Solution. Filter.

**FIXING SOLUTION**

To 250 mL of methanol, add 0.27 mL of formaldehyde, and dilute to 500.0 mL with water.

**SILVER NITRATE REAGENT**

To a mixture of 3 mL of concentrated ammonia and 40 mL of 1 M sodium hydroxide, add 8 mL of a 200 g/L solution of silver nitrate, dropwise, with stirring. Dilute to 200 mL with water.
DEVELOPER SOLUTION

Dilute 2.5 mL of a 20 g/L solution of citric acid and 0.27 mL of formaldehyde to 500.0 mL with water.

BLOCKING SOLUTION

A 10% (v/v) solution of acetic acid.

Coomassie Staining

Immerse the gel in a large excess of Coomassie Staining Solution, and allow to stand for at least 1 h. Remove the staining solution.

Destain the gel with a large excess of Destaining Solution. Change the Destaining Solution several times until the stained protein bands are clearly distinguishable on a clear background. The more thoroughly the gel is destained, the smaller is the amount of protein that can be detected by the method. More rapid destaining can be achieved by including a few grams of anion-exchange resin or a small sponge in the Destaining Solution. [NOTE—The acid–alcohol solutions used in this procedure do not completely fix proteins in the gel. This can lead to losses of some low-molecular-mass proteins during the staining and destaining of thin gels. Permanent fixation is obtainable by allowing the gel to stand in a mixture of 1 volume of trichloroacetic acid, 4 volumes of methanol, and 5 volumes of water for 1 h before it is immersed in the Coomassie Staining Solution.]

Silver Staining

Immerse the gel in a large excess of Fixing Solution, and allow it to stand for 1 h. Remove the Fixing Solution, add fresh Fixing Solution, and incubate for at least 1 h or overnight, if convenient. Discard the Fixing Solution, and wash the gel in a large excess of water for 1 h. Soak the gel for 15 min in a 1% (v/v) solution of glutaraldehyde. Wash the gel twice for 15 min in a large excess of water. Soak the gel in fresh Silver Nitrate Reagent for 15 min, in darkness. Wash the gel three times for 5 min in a large excess of water. Immerse the gel for about 1 min in Developer Solution until satisfactory staining has been obtained. Stop the development by incubation in the Blocking Solution for 15 min. Rinse the gel with water.

RECORDING THE RESULTS

Gels are photographed or scanned while they are still wet or after an appropriate drying procedure. Currently, gel-scanning systems with data analysis software are commercially available to photograph and analyze the wet gel immediately.

Depending on the staining method used, gels are treated in a slightly different way. For Coomassie staining, after the destaining step, allow the gel to stand in a 100 g/L solution of glycerol for at least 2 h (overnight incubation is possible). For silver staining, add to the final rinsing a step of 5 min in a 20 g/L solution of glycerol.

Drying of stained SDS polyacrylamide gels is one of the methods to have permanent documentation. This method frequently results in gel cracking during drying between cellulose films.

Immerse two sheets of porous cellulose film in water, and incubate for 5–10 min.
Place one of the sheets on a drying frame. Carefully lift the gel, and place it on the cellulose film. Remove any trapped air bubbles, and pour a few mL of water around the edges of the gel. Place the second sheet on top, and remove any trapped air bubbles. Complete the assembly of the drying frame. Place in an oven or leave at room temperature until dry.

MOLECULAR MASS DETERMINATION

Molecular masses of proteins are determined by comparison of their mobilities with those of several marker proteins of known molecular weight. Mixtures of prestained and unstained proteins with precisely known molecular masses blended for uniform staining are available for calibrating gels. They are available in various molecular mass ranges. Concentrated stock solutions of proteins of known molecular mass are diluted in the appropriate sample buffer and are loaded on the same gel as the protein sample to be studied.

Immediately after the gel has been run, mark the position of the bromophenol blue tracking dye to identify the leading edge of the electrophoretic ion front. This can be done by cutting notches in the edges of the gel or by inserting a needle soaked in India ink into the gel at the dye front. After staining, measure the migration distances of each protein band (markers and unknowns) from the top of the resolving gel. Divide the migration distance of each protein by the distance traveled by the tracking dye. The normalized migration distances are referred to as the relative mobilities of the proteins (relative to the dye front), or $R_f$. Construct a plot of the logarithm of the relative molecular masses ($M_i$) of the protein standards as a function of the $R_f$ values. Unknown molecular masses can be estimated by linear regression analysis (more accurately, by nonlinear regression analysis) or interpolation from the curves of log $M_i$ against $R_f$ if the values obtained for the unknown samples are positioned along the approximately linear part of the graph.

VALIDATION OF THE TEST

The test is not valid unless the target resolution range of the gel has been demonstrated by the distribution of appropriate molecular mass markers, e.g., across 80% of the length of the gel. The separation obtained for the expected proteins must show a linear relationship between the logarithm of the molecular mass and the $R_f$. If the plot has a sigmoidal shape, then only
data from the linear region of the curve can be used in the calculations. Additional validation requirements with respect to the test sample may be specified in individual monographs.

Sensitivity also must be validated. A reference protein control corresponding to the desired concentration limit that is run in parallel with the test samples can serve as a system suitability check of the experiment.

**QUANTITATION OF IMPURITIES**

SDS-PAGE is often used as a limit test for impurities. When impurities are quantitated by normalization to the main band using an integrating densitometer or image analysis, the responses must be validated for linearity. Note that depending on the detection method and protein, as described in the introduction of the section Detection of Proteins in Gels, the linear range can vary but can be assessed within each run by using one or more control samples containing an appropriate range of protein concentrations.

When the impurity limit is specified in the individual monograph, analysts should prepare a reference solution corresponding to that level of impurity by diluting the test solution. For example, when the limit is 5%, a reference solution would be a 1:20 dilution of the test solution. No impurity (any band other than the main band) in the electropherogram obtained with the test solution may be more intense than the main band obtained with the reference solution.

Under validated conditions, impurities can be quantified by normalization to the main band, using an integrating densitometer, or by image analysis.

**〈1057〉 BIOTECHNOLOGY-DERIVED ARTICLES—TOTAL PROTEIN ASSAY**

**Delete the following:**

*This chapter provides guidance and procedures used for characterization of biotechnology-derived articles. This chapter is harmonized with the corresponding chapter in *JP* and *EP*. Other characterization tests, also harmonized, are provided in *Biotechnology-Derived Articles—Amino Acid Analysis* (1052), *Capillary Electrophoresis* (1053), *Biotechnology-Derived Articles—Isoelectric Focusing* (1054), *Biotechnology-Derived Articles—Peptide Mapping* (1055), and *Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis* (1056).* (USP 1-Aug-2019)

**Change to read:**

**INTRODUCTION**

A number of factors needs to be considered when choosing a procedure for the measurement of total protein content in a pharmacopeial preparation. Those factors include the complexity of the sample, constraints that impact the procedure, the required accuracy/variability of the procedure, and the desired analyst contact or automation associated with the procedure (e.g., sample availability, analysis time). The choice of a total protein measurement procedure balances these and other factors to meet the application need.

An analytical separation technique (e.g., HPLC, capillary electrophoresis) may be the best option as a total protein measurement procedure when analyzing a complex sample. Separating the sample into various components and integrating relevant peaks on a chromatogram or electropherogram can be used to manage 1) sample components that interfere with spectrophotometric protein measurements; 2) a mixture of proteins or peptides in the sample, including excipients (e.g., human serum albumin), where the individual protein components need to be quantitated; 3) other sample attributes that are being assessed (e.g., identity, impurity quantitation) during the analysis; 4) a limited sample quantity available for analysis; or 5) the balance between analysis time, instrument complexity, and other factors that favor a separation procedure over a spectrophotometric procedure.

Amino acid quantitation may also be suitable for quantitation of total protein and is often used as a primary method of calibration for other total protein measurement procedures (e.g., determination of an extinction coefficient used with protein absorbance at 280 nm).

Spectrophotometric procedures for total protein measurement are often employed for the analysis of pharmacopeial preparations due to their simplicity, high sample throughput for a given analysis time, and low cost. (USP 1-Aug-2019)

Many of the total protein assay methods described below can be performed successfully using kits from commercial sources. (USP 1-Aug-2019)

**Add the following:**

**SAMPLE EXTRACTION METHODS FOR TOTAL PROTEIN ASSAYS**

Accurate measurement of total protein cannot always be accomplished if there are interfering substances in the sample. This interference can be observed with spectrophotometric methods of total protein measurement. In order to accurately measure the total protein content in samples with these interfering substances, a method to remove these substances needs to be part of the procedure and the total procedure (extraction in combination with a measurement method) should be validated as suitable for its intended purpose.
It is possible that an interfering substance exhibits a known reproducible effect on the assay that can be eliminated by using an appropriate blank containing the interfering substance to calibrate the spectrophotometer to zero. As an alternative, a spike control added to the Test solution or sample matrix can be used to adjust the test result based on the spike recovery.

Sample extraction techniques often involve precipitating protein from the sample, leaving the interfering substances in the liquid phase that is subsequently removed, and then solubilizing the precipitated protein in the assay buffer for analysis. Precipitating protein from the sample can also have the advantage of concentrating proteins from a dilute solution, thereby allowing a sample that was too dilute to use with a particular spectrophotometric procedure to now be within the useful range of the method. Gel filtration procedures to remove an interfering substance can result in sample dilution which should also be considered when developing the method of analysis. With all techniques, volumes of solutions used must be measured quantitatively to allow accurate calculation of concentration and dilution factors that are used in the final calculation of protein concentration in the starting sample.

The use of an extractive procedure may introduce a sample preparation error and affect the precision of the protein concentration result. When an extraction procedure is performed on Test solutions, it should also be performed for Standard solutions, system suitability solutions, and the Blank. Samples should be homogeneous and free of particles. Examples of several possible sample extraction procedures, depending upon the nature of the interfering substance, are described below but others may be demonstrated as suitable for use too.

**Procedure 1—Protein Precipitation with Acetone**

1. Add acetone to the sample to bring the concentration of acetone to 85%-90%.
2. Let the sample stand for 1 h. If necessary for appropriate recovery, hold the sample at 4°C overnight.
3. Centrifuge the sample at 14,000 × g or greater for 10 min. Discard the supernatant.
4. Dissolve the pellet in the appropriate buffer for analysis.

**Procedure 2—Protein Precipitation with Trichloroacetic Acid**

**SODIUM DEOXYCHOLATE REAGENT**

Prepare a solution of 1.5 g/L sodium deoxycholate in water.

**TCA REAGENT**

Prepare a solution of 720 g/L trichloroacetic acid (TCA) in water.

**PROCEDURE**

1. Add 0.1 mL of Sodium deoxycholate reagent to 1.0 mL of Test solution.
2. Mix using a vortex mixer, and then incubate for 10 min.
3. Add 0.1 mL of TCA reagent, and mix on a vortex mixer.
4. Centrifuge at a minimum of 6700 × g for 30 min.
5. Discard the supernatant.
6. Dissolve the protein pellet in 1.0 mL of the appropriate buffer for analysis.

**Procedure 3—Solid Phase Device Extraction**

**BUFFER PREPARATION**

Prepare the appropriate buffer or solvent mixture depending upon the solid phase extraction (SPE) device being used for fractionation.

**PROCEDURE**

1. Load the sample onto the SPE device.
2. Wash with a suitable buffer or solvent mixture.
3. Elute the protein with an appropriate buffer or solvent mixture.

**Procedure 4—Column Gel Filtration**

**BUFFER PREPARATION**

Prepare the appropriate buffer for the test method.
PROCEDURE

The protein is purified using either gravity or a centrifugal procedure per the manufacturer’s instructions.\(^1\) (USP 1-Aug-2019)

Change to read:

\^{TOTAL PROTEIN MEASUREMENT PROCEDURES}

Ideally, the protein in the Standard solution that is used to create the calibration curve should be the same protein as in the Test solution. This may not be practical and the use of a generic protein in the Standard solution may be substituted. Bovine serum albumin is often used because it is readily available and can be purchased at high purity. However, using a generic protein to create the standard curve may not be the ideal choice for all assays depending on the principle of the assay and the protein structure responsible for the response. Due to the potential variation in responses between different proteins that may be used to create the standard curve, the protein used for the standard curve should always be reported. In addition (unless otherwise specified in the individual monograph) prepare the Reference Standard or reference material for the protein under test in the same buffer used to prepare the Test solution. (USP 1-Aug-2019)

Method 1

Protein in solution absorbs UV light at a wavelength of 280 nm due to the presence of aromatic amino acids, mainly tyrosine and tryptophan. This property is the basis of Method 1. If the buffer used to solubilize the protein has a high absorbance relative to water, there is an interfering substance in the buffer. This interference can be compensated for when the spectrophotometer is adjusted to zero buffer absorbance. The results may be compromised if the interference results in a large absorbance that challenges the limit of sensitivity of the spectrophotometer. Furthermore, at low concentrations protein can be absorbed onto the cuvette, thereby reducing the content in solution. This can be prevented by preparing samples at higher concentrations or by using a nonionic detergent in the preparation.

\(\text{To determine whether the protein sample is contaminated with nucleic acid, a 260/280 ratio is recommended if no other methods are used to quantitate the nucleic acids. Table 1 can be used as a guide for protein purity.}\)

\*\*Table 1. 260/280 Ratio for Estimating Nucleic Acid Contamination and Protein Purity of the Sample

<table>
<thead>
<tr>
<th>Protein (%)</th>
<th>Nucleic Acid (%)</th>
<th>260/280 Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>0</td>
<td>0.57</td>
</tr>
<tr>
<td>95</td>
<td>5</td>
<td>1.06</td>
</tr>
<tr>
<td>90</td>
<td>10</td>
<td>1.32</td>
</tr>
<tr>
<td>70</td>
<td>30</td>
<td>1.73</td>
</tr>
</tbody>
</table>

TEST SOLUTION

Dissolve a suitable quantity of the protein under test in the appropriate buffer to obtain a solution having a concentration of 0.2–2 mg/mL.

STANDARD SOLUTION

Unless otherwise specified in the individual monograph, prepare a solution of Reference Standard or reference material for the protein under test in the same buffer and at the same concentration as the Test solution.

PROCEDURE

Concomitantly determine the \(\text{absorbance values}\) of the Standard solution and Test solution in quartz cells at a wavelength of 280 nm with a suitable spectrophotometer (see Ultraviolet-Visible Spectroscopy (857)), using the buffer as the Blank. \(\text{[NOTE—Keep the Test solution, Standard solution, and buffer at the same temperature during testing.]}\) To obtain accurate results, the response should be linear in the range of protein concentrations to be assayed.

LIGHT SCATTERING

The accuracy of the UV spectroscopic determination of protein can be decreased by the scattering of light by the Test solution if the proteins in solution exist as particles comparable in size to the wavelength of the measuring light (250–300 nm), scattering of the light beam results in an apparent increase in absorbance of the test specimen. To calculate the absorbance at 280 nm due to light scattering, determine the \(\text{absorbance values}\) of the Test solution at wavelengths of 320, 325, 330, 335, 340, 345, and 350 nm. Using the linear regression method, plot the log of the observed absorbance versus the log of the wavelength, and determine the standard curve best fitting the plotted points. From the graph

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1 Suitable commercial columns are available. For example, PD-10 columns (available from GE Healthcare) for samples ranging from 1.0–2.5 mL, NAP-10 columns for samples up to 1.0 mL, NAP-S columns for samples up to 0.5 mL, or another suitable alternative.
so obtained, extrapolate the absorbance value due to light scattering at 280 nm. Subtract the absorbance due to light scattering from the total absorbance at 280 nm to obtain the absorbance value of the protein in solution. Filtration with a filter having a 0.2-µm porosity or clarification by centrifugation may be performed to reduce the effect of light scattering, especially if the solution is noticeably turbid.

**CALCULATIONS**

Calculate the concentration, \( C_{ls} \), of protein in the test specimen by the formula:

\[
\text{Result} = C_{ls}(A_{ls}/A_{ls})
\]

in which \( C_{ls} \) is the concentration of the **Standard solution**; and \( A_{ls} \) and \( A_{ls} \) are the corrected absorbance values of the **Test solution** and the **Standard solution**, respectively (see (857)).

**Method 2**

This method, commonly referred to as the Lowry assay, is based on the reduction by protein of the phosphomolybdic–tungstic mixed acid chromogen in the Folin-Ciocalteu’s phenol reagent, resulting in an absorbance maximum at 750 nm. The Folin-Ciocalteu’s phenol reagent reacts primarily with tyrosine residues in the protein, which can lead to variation in the response of the assay to different proteins. Because the method is sensitive to interfering substances, a procedure for precipitation of the protein from the test specimen may be used. Most interfering substances cause a lower color yield; however, some detergents cause a slight increase in color. A high salt concentration may cause a precipitate to form. Because different protein species may give different color response intensities, the standard protein and test protein should be the same.

The effect of interfering substances can be minimized by dilution, provided the concentration of the protein under test remains sufficient for accurate measurement. Alternatively, procedures described in Sample Extraction Methods for Total Protein Assays could be used to remove interfering substances from the test sample.

The Lowry assay can be used for protein concentrations of 5–100 µg/mL. Wider ranges may be acceptable if the range of standard concentrations selected results in a linear curve.

**STANDARD SOLUTIONS**

Unless otherwise specified in the individual monograph, the Reference Standard or reference material for the protein under test in the buffer used to prepare the **Test solution**. Dilute portions of this solution with the same buffer to obtain NLT 5 **Standard solutions** having concentrations between 5 and 100 µg of protein per mL, the concentrations being evenly spaced.

**TEST SOLUTION**

Dissolve a suitable quantity of the protein under test in the appropriate buffer to obtain a solution having a concentration within the range of the concentrations of the **Standard solutions**. An appropriate buffer will produce a pH in the range of 10.0–10.5.

**BLANK**

Use the buffer used to prepare the **Test solution** and the **Standard solutions**.

**REAGENTS AND SOLUTIONS**

- **Copper sulfate reagent**: Dissolve 100 mg of cupric sulfate and 200 mg of sodium tartrate in water, dilute with water to 50 mL, and mix. Dissolve 10 g of sodium carbonate in water to a final volume of 50 mL, and mix. Slowly pour the sodium carbonate solution into the copper sulfate solution with mixing. Prepare this solution fresh daily.

- **SDS solution**: Dissolve 5 g of sodium dodecyl sulfate in water, and dilute with water to 100 mL.

- **Sodium hydroxide solution**: Dissolve 3.2 g of sodium hydroxide in water, dilute with water to 100 mL, and mix.

- **Alkaline copper reagent**: Prepare a mixture of Copper sulfate reagent, SDS solution, and Sodium hydroxide solution (1:2:1). This reagent may be stored at room temperature for up to 2 weeks.

- **Diluted Folin-Ciocalteu’s phenol reagent**: Mix 10 mL of Folin-Ciocalteu’s phenol TS with 50 mL of water. Store in an amber bottle at room temperature.

**PROCEDURE**

To 1 mL of each **Standard solution**, the **Test solution**, and the **Blank**, add 1 mL of Alkaline copper reagent, and mix. Allow to stand at room temperature for 10 min. Add 0.5 mL of the Diluted Folin-Ciocalteu’s phenol reagent to each solution, mix each tube immediately, and hold at room temperature for about 30 min.

**[NOTE—Color development reaches a maximum in 20–30 min during incubation at room temperature, after which there is a gradual loss of color.]**

Determine the absorbance values of the **Standard solutions** and **Test solution** at 750 nm with a suitable spectrophotometer, using the solution from the **Blank** to set the instrument to zero (see (857)).
Determine the absorbance values for all substances. When interfering substances are present, their effect may be minimized by dilution, provided that the concentration range is sufficiently small, it will approach linearity. Using the linear regression method, plot the absorbance values of the solutions from the Standard solutions versus the protein concentrations, and determine the standard curve best fitting the plotted points. From the standard curve so obtained and the absorbance of the Test solution, determine the concentration of protein in the Test solution.

**Method 3**

This method, commonly referred to as the Bradford assay, is based on the absorption shift from 470 nm to 595 nm observed when the Brilliant Blue G dye binds to protein. The Brilliant Blue G dye binds most readily to arginyl and lysyl residues in the protein, which can lead to variation in the response of the assay to different proteins. There are relatively few interfering substances, but detergents and ampholytes in the test specimen should be avoided. Highly alkaline specimens may interfere with the acidic reagent.

**STANDARD SOLUTIONS**

Unless otherwise specified in the individual monograph, solubilize the Reference Standard or reference material for the protein under test in the buffer used to prepare the Test solution. Dilute portions of this solution with the same buffer to obtain NLT 5 Standard solutions having concentrations of 100 µg–1 mg/mL of protein, the concentrations being evenly spaced.

**TEST SOLUTION**

Solubilize a suitable quantity of the protein under test in the appropriate buffer to obtain a solution having a concentration within the range of the concentrations of the Standard solutions.

**BLANK**

Use the buffer used to prepare the Test solution and the Standard solutions.

**COOMASSIE REAGENT**

Dissolve 100 mg of Brilliant Blue G in 50 mL of alcohol. [Note—Not all dyes have the same Brilliant Blue G content, and different products may give different results.] Add 100 mL of phosphoric acid, dilute with water to 1 L, and mix. Pass the solution through filter paper (Whatman #1 or equivalent), and store the filtered reagent in an amber bottle at room temperature. [Note—Slow precipitation of the dye will occur during storage of the reagent. Filter the reagent before use.]

**PROCEDURE**

Add 5 mL of the Coomassie reagent to 100 µL of each Standard solution, the Test solution, and the Blank, and mix by inversion. Avoid foaming, which will lead to poor reproducibility. Incubate at room temperature for a suitable period of time, and then determine the absorbance values of the solutions from the Standard solutions and Test solution at 595 nm with a suitable spectrophotometer (see (857)), using the Blank to set the instrument to zero. [Note—Do not use quartz (silica) spectrophotometer cells: the dye binds to this material. Because different protein species may give different color response intensities, the standard protein and test protein should be the same. For consistent and accurate results, the absorbance values for all Standard solutions, Blank, and Test solutions should be determined within a suitable period of time to assure reproducible results of adding the Coomassie reagent.]

**CALCULATIONS**

[Note—The relationship of absorbance to protein concentration is nonlinear; however, if the standard curve concentration range is sufficiently small, it will approach linearity.] Plot the absorbance values of the Standard solutions versus the protein concentrations, and use linear regression to establish the standard curve. Determine the concentration of protein in the Test solution from the standard curve and the absorbance of the Test solution.

**Method 4**

This method, commonly referred to as the bicinchoninic acid (BCA) assay, is based on reduction of the cupric (Cu²⁺) ion to cuprous (Cu¹⁺) ion by protein. The bicinchoninic acid reagent is used to detect the cuprous ion. The method has few interfering substances. When interfering substances are present, their effect may be minimized by dilution, provided that the concentration of the protein under test remains sufficient for accurate measurement. If substances that will cause interference in the test are

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2 Dye purity is important in the reagent preparation. Serva Blue G (Crescent Chemical Company, Islandia, NY) is an acceptable grade.
present, proceed as directed in Sample Extraction Methods for Total Protein Assays. Because different protein species may give different color response intensities, the standard protein and test protein should be the same.

**STANDARD SOLUTIONS**

Unless otherwise specified in the individual monograph, the Reference Standard or reference material for the protein under test in the buffer used to prepare the Test solution. Dilute portions of this solution with the same buffer to obtain NLT 5 Standard solutions having concentrations of 10–1200 µg/mL of protein, the concentrations being evenly spaced.

**TEST SOLUTION**

Dissolve a suitable quantity of the protein under test in the appropriate buffer to obtain a solution having a concentration within the range of the concentrations of the Standard solutions.

**BLANK**

Use the buffer used to prepare the Test solution and the Standard solutions.

**REAGENTS**

**BCA reagent**: Dissolve about 10 g of bicinechonic acid, 20 g of sodium carbonate monohydrate, 1.6 g of sodium tartrate, 4 g of sodium hydroxide, and 9.5 g of sodium bicarbonate in water. Adjust, if necessary, with sodium hydroxide or sodium bicarbonate to a pH of 11.25. Dilute with water to 1 L, and mix.

**Copper sulfate reagent**: Dissolve about 2 g of cupric sulfate in water to a final volume of 50 mL.

**Copper–BCA reagent**: Mix 1 mL of Copper sulfate reagent and 50 mL of BCA reagent.

**PROCEDURE**

Mix 0.1 mL of each Standard solution, the Test solution, and the Blank with 2 mL of the Copper–BCA reagent. Incubate the solutions at 37° for 30 min, note the time, and allow the mixtures to cool to room temperature. Within 60 min following the incubation time, determine the absorbance values for the Standard solutions and the Test solution at 562 nm with a suitable spectrophotometer (see 857), using the Blank to set the instrument calibration to zero. The color intensity continues to increase gradually after the solutions are cooled to room temperature.

**CALCULATIONS**

[NOTE—The relationship of absorbance to protein concentration is nonlinear; however, if the standard curve concentration range is sufficiently small, it will approach linearity.] Plot the absorbance values of the solutions from the Standard solutions versus the protein concentrations and determine the standard curve best fitting the plotted points. Determine the concentration of protein in the Test solution from the standard curve and the absorbance of the Test solution.

**Method 5**

This method, commonly referred to as the Biuret assay, is based on the interaction of cupric (Cu²⁺) ion with protein in an alkaline solution and the development of absorbance at 545 nm. This test shows minimal difference between IgG and albumin samples at the same concentration. Addition of the sodium hydroxide and the Biuret reagent as a combined reagent, insufficient mixing after the addition of the sodium hydroxide, or an extended time between the addition of the sodium hydroxide solution and the addition of the Biuret reagent will give IgG samples a higher response than albumin samples. The trichloroacetic acid method (described in Procedure 2 of Sample Extraction Methods for Total Protein Assays) used to minimize the effects of interfering substances can also be used to determine the protein content in test specimens at concentrations below 500 µg/mL.

**STANDARD SOLUTIONS**

Prepare the Reference Standard or reference material for the protein under test in 0.9% sodium chloride solution. Dilute portions of this solution with 0.9% sodium chloride solution to obtain NLT 3 Standard solutions having concentrations of 0.5–10 mg/mL, the concentrations being evenly spaced.

**TEST SOLUTION**

Prepare a solution of the test protein in 0.9% Sodium chloride solution having a concentration within the range of the concentrations of the Standard solutions.
Test solution

A solution that develops turbidity or a precipitate is not acceptable for calculation of protein concentration.

**Fluorescence Spectroscopy**

**Min.** Add 3 mL of 0.5 N sodium hydroxide, and mix. Using a suitable fluorometer (see **Biuret reagent**), determine the absorbance of the standard curve. Calculate the correlation coefficient for the line.

Plot the absorbance values versus the protein concentrations and use linear regression to establish the standard curve and the absorbance of the Test solution.

**Calculations**

Within the given range of the standards, the relationship of absorbance to protein concentration is approximately linear. Plot the absorbance values of the Standard solutions versus the protein concentrations and use linear regression to establish the standard curve. Calculate the correlation coefficient for the line. A suitable system is one that yields a line having a correlation coefficient of NLT 0.99. Determine the concentration of protein in the Test solution from the standard curve and the absorbance of the Test solution.

**Method 6**

This fluorometric method is based on the derivatization of the protein with o-phthalaldehyde (OPA), which reacts with the primary amines of the protein (i.e., NH₂-terminal amino acid and the ε-amino group of the lysine residues). The sensitivity of the test can be increased by hydrolyzing the protein before testing. Hydrolysis makes the ε-amino group of the constituent amino acids of the protein available for reaction with the OPA reagent. The method requires very small quantities of the protein.

Primary amines, such as tris(hydroxymethyl)aminomethane and amino acid buffers, react with OPA and must be avoided or removed. Ammonia at high concentrations will react with OPA as well. The fluorescence obtained when amine reacts with OPA can be unstable. The use of automated procedures to standardize the analysis may improve the accuracy and precision of the test.

**Standard solutions**

Unless otherwise specified in the individual monograph, prepare the Reference Standard or reference material for the protein under test in the buffer used to prepare the Test solution. Dilute portions of this solution with the same buffer to obtain NLT 5 Standard solutions having concentrations of 10–200 µg/mL of protein, the concentrations being evenly spaced.

**Test solution**

Solubilize a suitable quantity of the protein under test in the appropriate buffer to obtain a solution having a concentration within the range of the concentrations of the Standard solutions.

**Blank**

Use the buffer used to prepare the Test solution and the Standard solutions.

**Reagents**

**Borate buffer**: Dissolve about 61.83 g of boric acid in water, and adjust with potassium hydroxide to a pH of 10.4. Dilute with water to 1 L, and mix.

**Stock OPA reagent**: Dissolve about 120 mg of OPA in 1.5 mL of methanol, add 100 mL of Borate buffer, and mix. Add 0.6 mL of polyoxyethylene (23) lauryl ether, and mix. This solution is stable at room temperature for at least 3 weeks.

**OPA reagent**: To 5 mL of Stock OPA reagent add 15 µL of 2-mercaptoethanol. Prepare at least 30 min prior to use. This reagent is stable for 1 day.

**Procedure**

Adjust each of the Standard solutions and the Test solution to a pH between 8.0 and 10.5. Mix 10 µL of the Test solution and each of the Standard solutions with 100 µL of OPA reagent, and allow to stand at room temperature for 15 min. Add 3 mL of 0.5 N sodium hydroxide, and mix. Using a suitable fluorometer (see Fluorescence Spectroscopy),
determine the fluorescent intensities of solutions from the Standard solutions and the Test solution at an excitation wavelength of 340 nm and an emission wavelength between 440–455 nm. [NOTE—The fluorescence of an individual specimen is read only once because irradiation decreases the fluorescent intensity.]

CALCULATIONS

The relationship of fluorescence to protein concentration is linear. Using the linear regression method, plot the fluorescent intensities of the solutions from the Standard solutions versus the protein concentrations, and determine the standard curve best fitting the plotted points. \(^\text{▲}\) Determine the concentration of protein in the Test solution from the standard curve and the fluorescence of the Test solution. \(^\text{▲}\) (USP 1-Aug-2019)

**Method 7**

This method is based on nitrogen analysis as a means of protein determination. Interference caused by the presence of other nitrogen-containing substances in the test specimen can affect the determination of protein by this method \(^\text{since}\) non-proteinaceous nitrogen will also be detected. \(^\text{▲}\) (USP 1-Aug-2019) Nitrogen analysis techniques destroy the protein under test and are not \(^\text{stand-alone methods}\) since they quantitate all nitrogen present. If either Procedure 1 or Procedure 2 below is used to determine protein content, then a suitable orthogonal method must also be used to verify that no other nitrogen-containing sources are present and contributing to the apparent total protein content. \(^\text{▲}\) (USP 1-Aug-2019)

**PROCEDURE 1**

Determine the nitrogen content of the protein under test as directed under Nitrogen Determination (461). Commercial instrumentation is available for the Kjeldahl nitrogen assay.

**PROCEDURE 2**

Commercial instrumentation is available for nitrogen \(^\text{(elemental)}\) analysis. Most nitrogen analysis instruments use pyrolysis (i.e., combustion of the sample in oxygen at temperatures approaching 1000\(^\circ\)C), which produces nitric oxide (NO) and similar oxides of nitrogen (NO\(_x\)) from the nitrogen present in the test protein. Some instruments convert the nitric oxides to nitrogen gas, which is quantified with a thermal conductivity detector. Other instruments mix nitric oxide (NO) with ozone (O\(_3\)) to produce excited nitrogen dioxide (NO\(_x\)), which emits light when it decays and can be quantified with a chemiluminescence detector. A protein reference material or reference standard that is relatively pure and is similar in composition to the test proteins is used to optimize the injection and pyrolysis parameters and to evaluate consistency in the analysis.

**CALCULATIONS**

The protein concentration is calculated by dividing the nitrogen content of the sample by the known nitrogen content of the protein. The known nitrogen content of the protein can be determined from the chemical composition of the protein or by comparison with the nitrogen content of the Reference Standard or reference material.
This general information chapter will discuss the selection of suitable chemical disinfectants and antiseptics; the demonstration of their bactericidal, fungicidal, and sporicidal efficacy; the application of disinfectants in the sterile pharmaceutical manufacturing area; and regulation and safety considerations. Biofilm formation and its relationship to disinfectants are outside the scope of this chapter. Additional information not covered in the chapter may be obtained from standard texts on disinfectants and antiseptics.¹

DEFINITIONS

**Antiseptic**—An agent that inhibits or destroys microorganisms on living tissue including skin, oral cavities, and open wounds.

**Chemical Disinfectant**—A chemical agent used on inanimate surfaces and objects to destroy infectious fungi, viruses, and bacteria, but not necessarily their spores. Sporicidal and antiviral agents may be considered a special class of disinfectants. Disinfectants are often categorized as high-level, intermediate-level, and low-level by medically oriented groups based upon their efficacy against various microorganisms.

**Cleaning Agent**—An agent for the removal from facility and equipment surfaces of product residues that may inactivate sanitizing agents or harbor microorganisms.

**Decontamination**—The removal of microorganisms by disinfection or sterilization.

**Disinfectant**—A chemical or physical agent that destroys or removes vegetative forms of harmful microorganisms when applied to a surface.

**Sanitizing Agent**—An agent for reducing, on inanimate surfaces, the number of all forms of microbial life including fungi, viruses, and bacteria.

**Sporicidal Agent**—An agent that destroys bacterial and fungal spores when used in sufficient concentration for a specified contact time. It is expected to kill all vegetative microorganisms.

**Sterilant**—An agent that destroys all forms of microbial life including fungi, viruses, and all forms of bacteria and their spores. Sterilants are liquid or vapor-phase agents.

Microorganisms differ greatly in their resistance to disinfectant agents. The order of resistance of clinically significant microorganisms to chemical disinfectants from most to least resistant is listed in Table 1.

### Table 1. The Resistance of Some Clinically Important Microorganisms to Chemical Disinfectants (Listed in Order of Decreasing Resistance)

<table>
<thead>
<tr>
<th>Type of Microorganisms</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacterial spores</td>
<td><em>Bacillus subtilis</em> and <em>Clostridium sporogenes</em></td>
</tr>
<tr>
<td>Mycobacteria</td>
<td><em>Mycobacterium tuberculosis</em></td>
</tr>
<tr>
<td>Nonlipid-coated viruses</td>
<td><em>Poliovirus</em> and <em>rhinovirus</em></td>
</tr>
<tr>
<td>Fungal spores and vegetative molds and yeast</td>
<td><em>Trichophyton</em>, <em>Cryptococcus</em>, and <em>Candida spp.</em></td>
</tr>
<tr>
<td>Vegetative bacteria</td>
<td><em>Pseudomonas aeruginosa</em>, <em>Staphylococcus aureus</em>, and <em>Salmonella spp.</em></td>
</tr>
<tr>
<td>Lipid-coated viruses</td>
<td><em>Herpes simplex virus</em>, <em>hepatitis B virus</em>, and <em>human immunodeficiency virus</em></td>
</tr>
</tbody>
</table>

CLASSIFICATION OF DISINFECTANTS

Chemical disinfectants are classified by their chemical type. This includes aldehydes, alcohols, halogens, peroxides, quaternary ammonium compounds, and phenolic compounds (see Table 2).

### Table 2. General Classification of Antiseptics, Disinfectants, and Sporicidal Agents

<table>
<thead>
<tr>
<th>Chemical Entity</th>
<th>Classification</th>
<th>Example</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aldehydes</td>
<td>Sporicidal agent</td>
<td>2% Glutaraldehyde</td>
</tr>
<tr>
<td>Alcohols</td>
<td>General purpose disinfectant, antiseptic, antiviral agent</td>
<td>70% Isopropyl alcohol, 70% alcohol</td>
</tr>
<tr>
<td>Chlorine and sodium hypochlorite</td>
<td>Sporicidal agent</td>
<td>0.5% Sodium hypochlorite</td>
</tr>
<tr>
<td>Phenolics</td>
<td>General purpose disinfectant</td>
<td>500 µg per g Chlorocresol, 500 µg per g chloroxylenol</td>
</tr>
<tr>
<td>Ozone</td>
<td>Sporicidal agent</td>
<td>8% Gas by weight</td>
</tr>
<tr>
<td>Hydrogen peroxide</td>
<td>Vapor phase sterilant, liquid sporicidal agent, antiseptic</td>
<td>4 µg per g H₂O₂ vapor, 10%--25% solution, 3% solution</td>
</tr>
<tr>
<td>Substituted diguanides</td>
<td>Antiseptic agent</td>
<td>0.5% Chlorhexidine gluconate</td>
</tr>
<tr>
<td>Peracetic acid</td>
<td>Liquid sterilant, vapor phase sterilant</td>
<td>0.2% Peracetic acid, 1 µg per g peracetic acid</td>
</tr>
<tr>
<td>Ethylene oxide</td>
<td>Vapor-phase sterilant</td>
<td>60 µg per g Ethylene oxide</td>
</tr>
</tbody>
</table>

The effectiveness of a disinfectant depends on its intrinsic biocidal activity, the concentration of the disinfectant, the contact time, the nature of the surface disinfected, the hardness of water used to dilute the disinfectant, the amount of organic materials present on the surface, and the type and the number of microorganisms present. Under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), the Environmental Protection Agency (EPA) registers chemical disinfectants marketed in the United States and requires manufacturers to supply product information on the use dilution, type of microorganisms killed, and the necessary contact time. Certain liquid chemical sterilizers intended for use on critical or semicritical medical devices are defined and regulated by the U.S. Food and Drug Administration (FDA).

### SELECTION OF AN ANTISEPTIC FOR HAND AND SURGICAL SITE DISINFECTION

Hands and surgical sites are disinfected in a hospital setting to reduce the resident flora and to remove transient flora (e.g., Staphylococcus pyogenes) and methicillin-resistant S. aureus and P. aeruginosa that have been implicated in hospital-associated infection. Use of antiseptics to disinfect hands has been shown to be more effective than soap and water in reducing the counts of bacteria on the skin; repeated antiseptic use further reduces these counts. These principles may be applied to clean-room operators in the pharmaceutical industry.

Common antiseptics include 4% chlorhexidine, 10% povidone–iodine, 3% hexachlorophene, 70% isopropyl alcohol, and 0.5% chlorhexidine in 95% alcohol.

### SELECTION OF A DISINFECTANT FOR USE IN A PHARMACEUTICAL MANUFACTURING ENVIRONMENT

When selecting a disinfectant for use in a pharmaceutical manufacturing area, the following points should be considered: the number and types of microorganisms to be controlled; the spectrum of activity of commercially available disinfectants; the claims as a sterilant; the disinfectant or sanitizer supported by the EPA registrations; the concentration, application method, and contact time of the disinfectant; the nature of the surface material being disinfected and its compatibility with the disinfectant; the amount of organic compounds on the surface that may inactivate a disinfectant; the possible need to maintain a residual bactericidal activity of the disinfectant on the surface; the corrosiveness of the disinfectant to equipment with repeated application; the safety considerations for operators applying the disinfectant; the compatibility of the disinfectant with cleaning agents and other disinfectants; the planned disinfectant rotation; and the steps that need to be taken to avoid the contamination of pharmaceutical products by a disinfectant.

### THEORETICAL DISCUSSION OF DISINFECTANT ACTIVITY

Plots of the log of the number of microorganisms per mL surviving in a disinfectant solution indicate that first-order kinetics can be applied as a gross approximation to the reduction in microbial count with respect to time. In practice, the plots show a more sigmoid curve with a slower initial reduction in numbers followed by an increasing rate with respect to time.

The rate constant, K, for the disinfection process can be calculated by the formula:

$$K = \frac{1}{t} \left( \log \frac{N_0}{N} \right)$$

in which $t$ is the time, in minutes, for the microbial count to be reduced from $N_0$ to $N$; $N_0$ is the initial number of organisms, in cfu per mL; and $N$ is the final number, in cfu per mL, of organisms.

As with a first-order chemical reaction, the same concentration of disinfectant reduces the number of organisms more rapidly at elevated temperatures. This can be expressed as a temperature, $T$, coefficient per $10^\circ$ rise in temperature, $Q_{10}$, calculated by the formula:

$$Q_{10} = \frac{T'}{T} - 10$$

in which $T'$ is $10^\circ$ more than $T$.

Further evidence that a first-order reaction is an inadequate description of disinfection is that the $Q_{10}$ values for chemical and enzyme reactions are 2 to 3, while the common disinfectants phenol and alcohol have a $Q_{10}$ of 4 and 45, respectively.

Critical to the successful employment of disinfectants is an understanding of the effect of disinfectant concentration on microbial reduction. A plot of the log of the time to reduce the microbial population in a standard inoculum to zero against the log of the disinfectant concentration is a straight line with the slope of the line termed the concentration exponent, $n$. The relationship can be expressed as follows:

$$
\text{Time to decontamination at } T' = \text{Time to decontamination at } T \\
\text{in which } T' = T + 10
$$

\[ n = \frac{\log (\text{kill time at concentration } C_2) - \log (\text{kill time at concentration } C_1)}{\log C_1 - \log C_2} \]

in which \( C_1 \) and \( C_2 \) are the higher and lower disinfectant concentrations, respectively.

The wide differences in concentration exponents, \( n \), have practical consequences in picking the use dilution of different disinfectants and in using dilution to neutralize a disinfectant in disinfectant-effectiveness testing and routine microbial monitoring of the manufacturing environment. For example, mercuric chloride has a concentration exponent of 1, so a 3-fold dilution will reduce the disinfectant activity by \( 3^1 \) (or by one-third), while phenol with a concentration exponent of 6 will have a 3\(^6\) (or a 729-fold) reduction in disinfectant activity. Disinfectants with a larger concentration exponent or dilution coefficient rapidly lose activity when diluted. The concentration exponents for some disinfectants are listed in Table 3.

### Table 3. Concentration Exponents of Common Antiseptics, Disinfectants, and Sterilants

<table>
<thead>
<tr>
<th>Disinfectant</th>
<th>Concentration Exponents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrogen peroxide</td>
<td>0.5</td>
</tr>
<tr>
<td>Sodium hypochlorite</td>
<td>0.5</td>
</tr>
<tr>
<td>Mercuric chloride</td>
<td>1</td>
</tr>
<tr>
<td>Chlorhexidine</td>
<td>2</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>1</td>
</tr>
<tr>
<td>Alcohol</td>
<td>9</td>
</tr>
<tr>
<td>Phenol</td>
<td>6</td>
</tr>
<tr>
<td>Quaternary ammonium compounds</td>
<td>0.8 to 2.5</td>
</tr>
<tr>
<td>Aliphatic alcohols</td>
<td>6.0 to 12.7</td>
</tr>
<tr>
<td>Phenolic compounds</td>
<td>4 to 9.9</td>
</tr>
</tbody>
</table>

Another important consideration may be the pH of the disinfectant. Many disinfectants are more active in the ionized form, while others are more active in the nonionized form. The degree of ionization will depend on the \( pK_a \) of the agent and the pH of the disinfection environment. For example, phenol, with a \( pK_a \) of 10, will be more effective at a pH below 7 where it is nonionized.

### MECHANISM OF DISINFECTANT ACTIVITY

Table 4 lists the sites and modes of action of some representative disinfectants.

### Table 4. Mechanism of Disinfectant Activity Against Microbial Cells

<table>
<thead>
<tr>
<th>Target</th>
<th>Disinfectant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell wall</td>
<td>Formaldehyde, hypochlorite, and glutaraldehyde</td>
</tr>
<tr>
<td>Cytoplasmic membrane, action on membrane potential</td>
<td>Anilides and hexachlorophene</td>
</tr>
<tr>
<td>Membrane enzymes, action on electron-transport chain</td>
<td>Hexachlorophene</td>
</tr>
<tr>
<td>Action on ATP</td>
<td>Chlorhexidine and ethylene oxide</td>
</tr>
<tr>
<td>Action on enzymes with −SH groups</td>
<td>Ethylene oxide, glutaraldehyde, hydrogen peroxide, hypochlorite, and iodine</td>
</tr>
<tr>
<td>Action on general membrane permeability</td>
<td>Alcohols, chlorhexidine, and quaternary ammonium compounds</td>
</tr>
<tr>
<td>Cell contents, general coagulation</td>
<td>Chlorhexidine, aldehydes, and quaternary ammonium compounds</td>
</tr>
<tr>
<td>Ribosomes</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>Nucleic acids</td>
<td>Hypochlorites</td>
</tr>
<tr>
<td>Thiol groups</td>
<td>Ethylene oxide, glutaraldehyde, hydrogen peroxide, and hypochlorite</td>
</tr>
<tr>
<td>Amino groups</td>
<td>Ethylene oxide, glutaraldehyde, and hypochlorite</td>
</tr>
<tr>
<td>General oxidation</td>
<td>Hypochlorite</td>
</tr>
</tbody>
</table>

### MICROBIAL RESISTANCE TO DISINFECTANTS

The development of microbial resistance to antibiotics is a well-described phenomenon. The development of microbial resistance to disinfectants is less likely to occur at significant levels, as disinfectants are more powerful biocidal agents than antibiotics. In addition, they are normally applied in high concentrations against low populations of microorganisms usually not growing actively, so the selective pressure for the development of resistance is less profound. However, the most frequently isolated microorganisms from an environmental monitoring program may be periodically subjected to use-dilution testing with the agents used in the disinfection program to confirm their susceptibility, as there are real differences among different species in resistance to the lethal effects of different sanitizers.
DISINFECTANT CHALLENGE TESTING

Under FIFRA, the EPA requires companies that register public health antimicrobial pesticide products including disinfectants, sanitization agents, sporicidal agents, and sterilants to ensure the safety and effectiveness of their products before they are sold or distributed. Companies registering these products must address the chemical composition of their product, include toxicology data to document that their product is safe if used as directed on the label, include efficacy data to document their claims of effectiveness against specific organisms and to support the directions for use provided in the labeling, and provide labeling that reflects the required elements for safe and effective use. While these directions provide valuable information, they may not be helpful in terms of the products’ use as disinfectants in a manufacturing environment.

In the United States, the official disinfectant testing methods are published by AOAC International\(^3\) and include the Phenol-Coefficient Test, Use-Dilution Method Test, Hard Surface Carrier Method, and Sporicidal Carrier Test. A scientific study submitted for EPA review in support of disinfectant registration must be conducted at a laboratory facility that follows the Good Laboratory Practices (GLP) regulations (21 CFR 58). To demonstrate the efficacy of a disinfectant within a pharmaceutical manufacturing environment, it may be deemed necessary to conduct the following tests: (1) use-dilution tests (screening disinfectants for their efficacy at various concentrations and contact times against a wide range of standard test organisms and environmental isolates); (2) surface challenge tests (using standard test microorganisms and microorganisms that are typical environmental isolates, applying disinfectants to surfaces at the selected use concentration with a specified contact time, and determining the log reduction of the challenge microorganisms); and (3) a statistical comparison of the frequency of isolation and numbers of microorganisms isolated prior to and after the implementation of a new disinfectant. This is considered necessary because critical process steps like disinfection of aseptic processing areas, as required by GMP regulations, need to be validated, and the EPA registration requirements do not address how disinfectants are used in the pharmaceutical, biotechnological, and medical device industries. For the surface challenge tests, the test organisms are enumerated using swabs, surface rinse, or contact plate methods. Neutralizers that inactivate the disinfectants should be included in either the diluent or microbiological media used for microbial enumeration or both. Information on disinfectant neutralization may be found in Validation of Microbial Recovery from Pharmacopeial Articles (1227).

The disinfectant efficacy test must have realistic acceptance criteria. In practice, sufficient organisms need to be inoculated on a 2-inch × 2-inch square of the surface being decontaminated, i.e., a coupon, to demonstrate at least a 2 (for bacterial spores) to 3 (for vegetative bacteria) log reduction during a predetermined contact time (i.e., 10 minutes over and above the recovery observed with a control disinfectant application). The efficacy of the neutralizers and their ability to recover inoculated microorganisms from the material should be demonstrated during the use-dilution or surface-challenge studies. Points to remember are that disinfectants are less effective against the higher numbers of microorganisms used in laboratory challenge tests than they are against the numbers that are found in clean rooms (see Microbiological Control and Monitoring of Aseptic Processing Environments (1116)); that inocula from the log growth phase that are typically employed in laboratory tests are more resistant, with the exception of spores formed during the static phase, than those from a static or dying culture or stressed organisms in the environment; and that microorganisms may be physically removed during actual disinfectant application in the manufacturing area.

Although not all inclusive, typical challenge organisms that may be employed are listed in Table 5.

<table>
<thead>
<tr>
<th>AOAC Challenge Organisms</th>
<th>Typical Environmental Isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bactericide: E. coli, ATCC 11229; S. aureus, ATCC 6538; P. aeruginosa, ATCC 15442</td>
<td>Bactericide: M. luteus, S. epidermidis, Coynobacterium jeikeium, P. vesiculosus</td>
</tr>
<tr>
<td>Fungicide: C. albicans, ATCC 10231 or 2091; Penicillium chrysogenum, ATCC 11709; A. brasiliensis, ATCC 16404</td>
<td>Fungicide: P. chrysogenum, A. brasiliensis</td>
</tr>
<tr>
<td>Sporicide: B. subtilis, ATCC 19659</td>
<td>Sporicide: B. sphaericus, B. thuringiensis</td>
</tr>
</tbody>
</table>

Because a wide range of different materials of construction are used in clean rooms and other controlled areas, each material needs to be evaluated separately to validate the efficacy of a given disinfectant. Table 6 contains a list of common materials used in clean room construction.

<table>
<thead>
<tr>
<th>Material</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stainless steel 304L and 316L grades</td>
<td>Work surfaces, filling equipment, and tanks</td>
</tr>
<tr>
<td>Glass</td>
<td>Windows and vessels</td>
</tr>
<tr>
<td>Plastic, vinyl</td>
<td>Curtains</td>
</tr>
<tr>
<td>Plastic, polycarbonate</td>
<td>Insulation coating</td>
</tr>
<tr>
<td>Lexan® (plexiglass)</td>
<td>Shields</td>
</tr>
<tr>
<td>Epoxy-coated gypsum</td>
<td>Walls and ceilings</td>
</tr>
<tr>
<td>Fiberglass-reinforced plastic</td>
<td>Wall paneling</td>
</tr>
<tr>
<td>Tyvek®</td>
<td>Equipment wraps</td>
</tr>
</tbody>
</table>

\(^3\) AOAC International Official Methods of Analysis, 15th, 16th, and 17th editions. Arlington, VA.
Table 6. Typical Surfaces to be Decontaminated by Disinfectants in a Pharmaceutical Manufacturing Area (continued)

<table>
<thead>
<tr>
<th>Material</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Terrazzo tiles</td>
<td>Floors</td>
</tr>
</tbody>
</table>

**DISINFECTANTS IN A CLEANING AND SANITIZATION PROGRAM**

The selection of suitable disinfectants and the verification of their effectiveness in surface challenge testing is critical in the development of a cleaning and sanitation program. Issues associated with the successful implementation of such a program are the development of written procedures, staff training, decisions on disinfectant rotation, institution of application methods and contact times, environmental monitoring to demonstrate efficacy, and personnel safety.

The cGMP 21 CFR 211.67, *Equipment Cleaning and Maintenance*, details the requirements for written procedures for cleaning, maintenance, and sanitization of pharmaceutical manufacturing equipment. These procedures should address the assignment of responsibility, establishment of schedules, details of cleaning operations, protection of clean equipment prior to use, inspection for cleanliness immediately prior to use, and maintenance of cleaning and sanitation records.

Staff involved in disinfection require training in microbiology, industry practices for cleaning and sanitization, safe handling of concentrated disinfectants, the preparation and disposal of disinfectants, and appropriate application methods. It should be emphasized that the preparation of the correct dilutions is critical because many disinfectant failures can be attributed to use of disinfectant solutions that are too dilute. Typically disinfectants used in aseptic processing and filling areas are diluted with Sterile Purified Water, and are prepared aseptically. Alternately, the disinfectant may be diluted with Purified Water, and then sterile filtered to eliminate microorganisms that may potentially persist in a disinfectant. Diluted disinfectants must have an assigned expiration dating justified by effectiveness studies.

The rotation of an effective disinfectant with a sporicide is encouraged. It is prudent to augment the daily use of a bactericidal disinfectant with weekly (or monthly) use of a sporicidal agent. The daily application of sporicidal agents is not generally favored because of their tendency to corrode equipment and because of the potential safety issues with chronic operator exposure. Other disinfection rotation schemes may be supported on the basis of a review of the historical environmental monitoring data. Disinfectants applied on potential product contact surfaces are typically removed with 70% alcohol wipes. The removal of residual disinfectants should be monitored for effectiveness as a precaution against the possibility of product contamination.

The greatest safety concerns are in the handling of concentrated disinfectants and the mixing of incompatible disinfectants. For example, concentrated sodium hypochlorite solutions (at a concentration of more than 5%) are strong oxidants and will decompose on heating, on contact with acids, and under the influence of light, producing toxic and corrosive gases including chlorine. In contrast, dilute solutions (at a concentration of less than 0.5%) are not considered as hazardous. Under no circumstances should disinfectants of different concentrations be mixed. Material Safety Data Sheets for all the disinfectants used in a manufacturing area should be available to personnel handling these agents. Appropriate safety equipment such as face shields, safety glasses, gloves, and uniforms must be issued to personnel handling the disinfectant preparation, and personnel must be trained in the proper use of this equipment. Safety showers and eye wash stations must be situated in the work area where disinfectant solutions are prepared.

1. **THE DISSOLUTION PROCEDURE: DEVELOPMENT AND VALIDATION**

**INTRODUCTION**

*Purpose*

The Dissolution Procedure: Development and Validation (1092) provides a comprehensive approach covering items to consider for developing and validating dissolution procedures and the accompanying analytical procedures. It addresses the use of automation throughout the test and provides guidance and criteria for validation. It also addresses the treatment of the data generated and the interpretation of acceptance criteria for immediate- and modified-release solid oral dosage forms.

*Scope*

Chapter (1092) addresses the development and validation of dissolution procedures, with a focus on solid oral dosage forms. Many of the concepts presented, however, may be applicable to other dosage forms and routes of administration. General recommendations are given with the understanding that modifications of the apparatus and procedures as given in USP general chapters need to be justified.

The organization of (1092) follows the sequence of actions often performed in the development and validation of a dissolution test. The sections appear in the following sequence.

1. **PRELIMINARY ASSESSMENT (FOR EARLY STAGES OF PRODUCT DEVELOPMENT/DISSOLUTION METHOD DEVELOPMENT)**
   1.1 Performing Filter Compatibility
   1.2 Determining Solubility and Stability of Drug Substance in Various Media

Published on March 26, 2020
1.3 Choosing a Medium and Volume
1.4 Choosing an Apparatus

2. METHOD DEVELOPMENT
2.1 Deaeration
2.2 Sinkers
2.3 Agitation
2.4 Study Design
   2.4.1 Time Points
   2.4.2 Observations
   2.4.3 Sampling
   2.4.4 Cleaning
2.5 Data Handling
2.6 Dissolution Procedure Assessment

3. ANALYTICAL FINISH
3.1 Sample Processing
3.2 Filters
3.3 Centrifugation
3.4 Analytical Procedure
3.5 Spectrophotometric Analysis
3.6 HPLC

4. AUTOMATION
4.1 Medium Preparation
4.2 Sample Introduction and Timing
4.3 Sampling and Filtration
4.4 Cleaning
4.5 Operating Software and Computation of Results
4.6 Common Deviations from the Compendia Procedures That May Require Validation

5. VALIDATION
5.1 Specificity/Placebo Interference
5.2 Linearity and Range
5.3 Accuracy/Recovery
5.4 Precision
   5.4.1 Repeatability of Analysis
   5.4.2 Intermediate Precision/Ruggedness
   5.4.3 Reproducibility
5.5 Robustness
5.6 Stability of Standard and Sample Solutions
5.7 Considerations for Automation

6. ACCEPTANCE CRITERIA
6.1 Immediate-Release Dosage Forms
6.2 Delayed-Release Dosage Forms
6.3 Extended-Release Dosage Forms
6.4 Multiple Dissolution Tests
6.5 Interpretation of Dissolution Results
   6.5.1 Immediate-Release Dosage Forms
   6.5.2 Delayed-Release Dosage Forms
   6.5.3 Extended-Release Dosage Forms

7. REFERENCES
to dissolve and can bias the results. Therefore, filtering the dissolution samples is usually necessary and should be done immediately if the filter is not positioned on the cannula.

Filtration also removes insoluble excipients that may otherwise interfere with the analytical finish. Selection of the proper filter material is important and should be accomplished, and experimentally justified, early in the development of the dissolution procedure. Important characteristics to consider when choosing a filter material are type, filter size, and pore size. The filter that is selected based on evaluation during the early stages of dissolution procedure development may need to be reconsidered at a later time point. Requalification has to be considered after a change in composition of the drug product or changes in the quality of the ingredients (e.g., particle size of microcrystalline cellulose).

Examples of filters used in dissolution testing can be cannula filters, filter disks or frits, filter tips, or syringe filters. The filter material has to be compatible with the media and the drug. Common pore sizes range from 0.20 to 70 μm, however, filters of other pore sizes can be used as needed. If the drug substance particle size is very small (e.g., micronized or nanoparticles), it can be challenging to find a filter pore size that excludes these small particles.

Adsorption of the drug(s) by the filter may occur and needs to be evaluated. Filter materials will interact with dissolution media to affect the recovery of the individual solutes and must be considered on a case-by-case basis. Different filter materials exhibit different drug-binding properties. Percentage of drug loss from the filtrate due to binding may be dependent on the drug concentration. Therefore the adsorptive interference should be evaluated on sample solutions at different concentrations bracketing the expected concentration range. Where the drug adsorption is saturable, discarding an initial volume of filtrate may allow the collection of a subsequent solution that approaches the original solution concentration. Alternative filter materials that minimize adsorptive interference can usually be found. Prewetting of the filter with the medium may be necessary. In addition, it is important that leachables from the filter do not interfere with the analytical procedure. This can be evaluated by analyzing the filtered dissolution medium and comparing it with the unfiltered medium.

The filter size should be based on the volume to be withdrawn and the amount of particles to be separated. Use of the correct filter dimensions will improve throughput and recovery, and also reduce clogging. Use of a large filter for small-volume filtration can lead to loss of sample through hold-up volume, whereas filtration through small filter sizes needs higher pressures and longer times, and the filters can clog quickly.

Filters used for USP Apparatus 4 need special attention because they are integrated in the flow-through process. Undissolved particles may deposit on the filters, creating resistance to the flow.

In the case of automated systems, selection of the filter with regard to material and pore size can be done in a similar manner to manual filtration. Flow rate through the filter and clogging may be critical for filters used in automated systems. Experimental verification that a filter is appropriate may be accomplished by comparing the responses for filtered and unfiltered standard and sample solutions. This is done by first preparing a suitable standard solution and a sample solution. For example, prepare a typical dissolution sample in a beaker and stir vigorously with a magnetic stirrer to dissolve the drug load completely. For standard solutions, compare the results for filtered solutions (after discarding the appropriate volume) to those for the unfiltered solutions. For sample solutions, compare the results for filtered solutions (after discarding the appropriate volume) to those for centrifuged, unfiltered solutions.

1.2 Determining Solubility and Stability of Drug Substance in Various Media

Physical and chemical characteristics of the drug substance need to be determined as part of the process of selecting the proper dissolution medium. When deciding the composition of the medium for dissolution testing, it is important to evaluate the influence of buffers, pH, and if needed, different surfactants on the solubility and stability of the drug substance. Solubility of the drug substance is usually evaluated by determining the saturation concentration of the drug in different media at 37°C using the shake-flask solubility method (equilibrium solubility). To level out potential ion effects between the drug and the buffers used in the media, mixtures of hydrochloric acid and sodium hydroxide are used to perform solubility investigations; this is in addition to the typical buffer solutions. In certain cases, it may be necessary to evaluate the solubility of the drug at temperatures other than 37°C (i.e., 25°C). The pH of the clear supernatant should be checked to determine whether the pH changes during the solubility test. Alternative approaches for solubility determination may also be used.

Typical media for dissolution may include the following (not listed in order of preference): diluted hydrochloric acid, buffers (phosphate or acetate) in the physiologic pH range of 1.2–7.5, simulated gastric or intestinal fluid (with or without enzymes), and water. For some drugs, incompatibility of the drug with certain buffers or salts may influence the choice of buffer. The molarity of the buffers and acids used can influence the solubilizing effect, and this factor may be evaluated.

Aqueous solutions (acidic or buffer solutions) may contain a percentage of a surfactant [e.g., sodium dodecyl sulfate (SDS), polysorbate, or lauryldimethylamine oxide] to enhance the solubility of the drug. The surfactants selected for the solubility investigations should cover all common surfactant types, i.e., anionic, nonionic, and cationic. When a suitable surfactant has been identified, different concentrations of that surfactant should be investigated to identify the lowest concentration needed to achieve sink conditions. Typically, the surfactant concentration is above its critical micellar concentration (CMC). Table 1 shows a list of some of the surfactants used in dissolution media. Approximate CMC values are provided with references when available. The list is not comprehensive and is not intended to exclude surfactants that are not listed. Other substances, such as cyclodextrins, have been used in dissolution media additives to enhance dissolution of poorly soluble compounds. The U.S. Food and Drug Administration (FDA) maintains a database of dissolution methods, including information on dissolution media that have been used (1). Typically, the amount of surfactant added is sufficient to achieve sink conditions in the desired volume of dissolution medium.

It is important to control the grade and purity of surfactants because use of different grades could affect the solubility of the drug. For example, SDS is available in both a technical grade and a high-purity grade. Obtaining polysorbate 80 from different sources can affect its suitability when performing high-performance liquid chromatography (HPLC) analysis.

There may be effects of counter-ions or pH on the solubility or solution stability of the surfactant solutions. For example, a precipitate forms when the potassium salt for the phosphate buffer is used at a concentration of 0.5 M in combination with SDS. This can be avoided by using the sodium phosphate salt when preparing media with SDS.
Routinely, the dissolution medium is buffered; however, the use of purified water as the dissolution medium is suitable for products with a dissolution behavior independent of the pH of the medium. There are several reasons why purified water may not be preferred. The water quality can vary depending on its source, and the pH of the water is not as strictly controlled as the pH of buffer solutions. Additionally, the pH can vary from day to day and can also change during the run, depending on the drug substance and excipients. Use of an aqueous–organic solvent mixture as a dissolution medium is discouraged; however, with proper justification this type of medium may be acceptable.

Investigations of the stability of the drug substance should be carried out, when needed, in the selected dissolution medium with excipients present, at 37°C. This elevated temperature has the potential to decrease solution stability (degradation). Stability should allow for sufficient time to complete or repeat the analytical procedure. Physical stability may be of concern when precipitation occurs because of lower solubility at room or refrigerated temperature.

### 1.3 Choosing a Medium and Volume

When developing a dissolution procedure, one goal is to have sink conditions, which are defined as having a volume of medium at least three times the volume required to form a saturated solution of drug substance. When sink conditions are present, it is more likely that dissolution results will reflect the properties of the dosage form. A medium that fails to provide sink conditions may be acceptable if it is appropriately justified. The composition and volume of dissolution medium are guided by the solubility investigations. For example, the choice and concentration of a surfactant need to be justified from the solubility data and the dissolution profiles.

The use of enzymes in the dissolution medium is permitted, in accordance with *Dissolution* (711), when dissolution failures occur as a result of cross-linking with gelatin capsules or gelatin-coated products. A discussion of the phenomenon of cross-linking and method development using enzymes can be found in *Capsules—Dissolution Testing and Related Quality Attributes* (1094). Validation should be performed with the method using enzymes according to section 5. Validation.

Another option is to use media that follow more closely the composition of fluids in the stomach and intestinal tract. These media may contain physiological surface-active ingredients, such as taurocholates. The media also may contain emulsifiers (lecithin) and components such as saline solution that increase osmolality. Also, the ionic strength or molarity of the buffer solutions may be manipulated. The media are designed to represent the fed and fasted state in the stomach and small intestine. These media may be very useful in modeling in vivo dissolution behavior of immediate-release (IR) dosage forms, in particular those containing lipophilic drug substances, and may help in understanding the dissolution kinetics of the product related to the physiological make up of the digestive fluids. Results of successful modeling of dissolution kinetics have been published, mainly for IR products. In the case of extended-release dosage forms with reduced effect of the drug substance on dissolution behavior, the use of such media needs to be evaluated differently. In vitro performance testing does not necessarily require media modeling the fasted and postprandial states (12, 13).

An acid stage is part of the testing of delayed-release products by *Method A* or *Method B* in (711). For drugs with acid solubility less than 10% of the label claim or drugs that degrade in acid the usefulness of the acid stage in detecting a coating failure is compromised. This would be handled on a case-by-case basis. Possible resolutions include the addition of surfactant to the acid stage, or adjustment of the specifications.

During selection of the dissolution medium, care should be taken to ensure that the drug substance is suitably stable throughout the analysis. In some cases, antioxidants such as ascorbic acid may be used in the dissolution medium to stabilize the drug. There are occasions where such actions are not sufficient. For compounds that rapidly degrade to form a stable degradant, monitoring the degradant alone or in combination with a drug substance may be more suitable than analyzing only.

---

Table 1. Commonly Used Surfactants with Critical Micelle Concentrations

<table>
<thead>
<tr>
<th>Surfactant</th>
<th>CMC (% wt/volume)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium dodecyl sulfate (SDS), Sodium lauryl sulfate (SLS)</td>
<td>0.18%–0.23%</td>
<td>(2–4)</td>
</tr>
<tr>
<td>Taurocholic acid sodium salt</td>
<td>0.2%</td>
<td>(3)</td>
</tr>
<tr>
<td>Cholic acid sodium salt</td>
<td>0.16%</td>
<td>(3)</td>
</tr>
<tr>
<td>Desoxycholic acid sodium salt</td>
<td>0.12%</td>
<td>(3)</td>
</tr>
<tr>
<td>Cetyltrimethyl ammonium bromide (CTAB, Hexadecyltrimethylammonium bromide)</td>
<td>0.033%–0.036% (0.92–1.0 mM)</td>
<td>(5, 6)</td>
</tr>
<tr>
<td>Benzethonium chloride (Hyamine 1622)</td>
<td>0.18% (4 mM)</td>
<td>(2)</td>
</tr>
<tr>
<td>Polysorbate 20 (Polyoxyethylene (20) sorbitan monolaureate, Tween 20)</td>
<td>0.07%–0.09%</td>
<td>(3, 7)</td>
</tr>
<tr>
<td>Polysorbate 80 (Polyoxyethylene (20) sorbitan monooleate, Tween 80)</td>
<td>0.02%–0.08%</td>
<td>(3, 7)</td>
</tr>
<tr>
<td>Caprylocapryl polyoxyyl-8 glycerides (Labrasol)</td>
<td>0.01%</td>
<td>(4)</td>
</tr>
<tr>
<td>Polysyrxol 35 castor oil (Cremophor EL)</td>
<td>0.02%</td>
<td>(8)</td>
</tr>
<tr>
<td>Polyoxyethylene 23 lauryl ether (Brij 35)</td>
<td>0.013%</td>
<td>(9)</td>
</tr>
<tr>
<td>Octoxinol (Triton X-100)</td>
<td>0.01%–0.03%</td>
<td>(3, 10)</td>
</tr>
<tr>
<td>Zwitterion</td>
<td>Lauryldimethylamine N-oxide (LDAO)</td>
<td>0.023%</td>
</tr>
</tbody>
</table>
the drug substance. In situ spectroscopic techniques tend to be less affected by degradation when compared with HPLC analysis (including UHPLC and other liquid chromatographic approaches).

For compendial Apparatus 1 (basket) and Apparatus 2 (paddle), the volume of the dissolution medium can vary from 500 to 1000 mL. Usually, the volume needed for the dissolution test can be determined in order to maintain sink conditions. In some cases, the volume can be increased to between 2 and 4 L using larger vessels and depending on the concentration and sink conditions of the drug; justification for this approach is expected. In practice, the volume of the dissolution medium is usually maintained within the compendial range given above. Alternatively, it may be preferable to switch to other compendial apparatus, such as a reciprocating cylinder (Apparatus 3), reciprocating holder (Apparatus 7), or flow-through cell (Apparatus 4). Certain applications may require low volumes of dissolution media (e.g., 100–200 mL) when the use of a paddle or basket is preferred. In these cases, an alternative, noncompendial apparatus (e.g., small-volume apparatus) may be used.

1.4 Choosing an Apparatus

The choice of apparatus is based on knowledge of the formulation design and the practical aspects of dosage form performance in the in vitro test system. In general, a compendial apparatus should be selected. For solid oral dosage forms, Apparatus 1 and Apparatus 2 are used most frequently. When Apparatus 1 or Apparatus 2 is not appropriate, another official apparatus may be used. Apparatus 3 (reciprocating cylinder) has been found especially useful for chewable tablets, soft gelatin capsules, delayed-release dosage forms, and nondisintegrating-type products, such as coated beads. Apparatus 4 (flow-through cell) may offer advantages for modified-release dosage forms and immediate-release dosage forms that contain active ingredients with limited solubility. In addition, Apparatus 4 may have utility for multiple dosage form types such as soft gelatin capsules, beaded products, suppositories, or depot dosage forms, as well as suspension-type extended-release dosage forms. Apparatus 5 (paddle over disk) and Apparatus 6 (rotating cylinder) are useful for evaluating and testing transdermal dosage forms. Apparatus 7 (reciprocating holder) has application to non-disintegrating, oral modified-release dosage forms, stents, and implants, as well as transdermal dosage forms. For semisolid dosage forms, the generally used apparatus include the vertical diffusion cell, immersion cell, and flow-through cell apparatus with the insert for topical dosage forms (see Semisolid Drug Products—Performance Tests (1724)).

Some changes can be made to the compendial apparatus; for example, a basket mesh size other than the typical 40-mesh basket (e.g., 10-, 20-, or 80-mesh) may be used when the need is clearly documented by supporting data. Care must be taken that baskets are uniform and meet the dimensional requirements specified in (711). A noncompendial apparatus may have some utility with proper justification, qualification, and documentation of superiority over the standard equipment. For example, a small-volume apparatus with mini paddles and baskets may be considered for low-dosage strength products. A rotating bottle or dialysis tubes may have utility for microspheres and implants, peak vessels, and modified flow-through cells for special dosage forms including powders and stents.

2. METHOD DEVELOPMENT

A properly designed test should yield data that are not highly variable, and should be free of significant stability problems. High variability in the results can make it difficult to identify trends or effects of formulation changes. Sample size can affect the observed variability. One guidance defines dissolution results as highly variable if the relative standard deviation (RSD) is more than 20% at time points of 10 min or less and more than 10% at later time points for a sample size of 12 (174). However, during method development, smaller sample sizes may be used, and the analyst will need to make a judgment accordingly. Most dissolution results, however, exhibit less variability. In the development of a dissolution procedure the source of the variability should be investigated, and attempts should be made to reduce variability whenever possible. The two most likely causes are the formulation itself (e.g., drug substance, excipients, or manufacturing process) or artifacts associated with the test procedure (e.g., coning, tablets sticking to the vessel wall or basket screen). Visual observations are often helpful for understanding the source of the variability and whether the dissolution test itself is contributing to the variability. Any time the dosage contents do not disperse freely throughout the vessel in a uniform fashion, aberrant results can occur. Depending on the problem, the usual remedies include changing any of the following factors: the apparatus type, speed of agitation, level of deaeration, sinker type, or composition of the medium.

Many causes of variability can be found in the formulation and manufacturing process. For example, poor content uniformity, process inconsistencies, excipient interactions or interference, film coating, capsule shell aging, and hardening or softening of the dosage form on stability may be sources of variability and interferences.

2.1 Deaeration

The significance of deaeration of the dissolution medium should be determined because air bubbles can act as a barrier to the dissolution process if present on the dosage unit or basket mesh and can adversely affect the reliability of the test results. Furthermore, bubbles can cause particles to cling to the apparatus and vessel walls. Bubbles on the dosage unit may increase buoyancy, leading to an increase in the dissolution rate, or may decrease the available surface area, leading to a decrease in the dissolution rate. Poorly soluble drugs are most sensitive to interference from air bubbles; therefore, deaeration may be needed when testing these types of products. A deaeration method is described as a footnote in the Procedure section of (711). Typical steps include heating the medium, filtering, and drawing a vacuum for a short period of time. Other methods of deaeration are available and are in routine use throughout the industry. Once a suitable deaeration process is identified, it should be documented as part of the dissolution procedure. The extent of deaeration can be evaluated by measuring the total dissolved gas pressure or by measuring the concentration of dissolved oxygen in water. For example, an oxygen concentration below 6 mg/L has been found effective as a marker for adequate deaeration of water for the Performance Verification Test with USP Prednisone Tablets RS.
Media containing surfactants usually are not deaerated because the process results in excessive foaming, and usually the effect of dissolved air on the dissolution process is mitigated by the reduced surface tension of the medium. Sometimes, deaerating the medium before adding surfactants can be effective.

To determine whether deaeration of the medium is necessary, compare results from dissolution samples run in non-deaerated medium and medium deaerated using a compendial technique, as described above. If no effect of deaeration is detected, this experiment could serve as justification that deaeration is not required in the future. If there is an effect, however, then it is necessary to carefully control this parameter, and it is prudent to characterize the robustness of the deaeration process. The dissolved gas content of deaerated media under atmospheric pressure is unstable and will tend toward saturation. Manipulations of the deaerated medium such as stirring or pouring can increase the rate at which atmospheric gases are redissolved.

2.2 Sinkers

Sinkers are often used to adjust the buoyancy of dosage forms that would otherwise float during testing with Apparatus 2. When sinkers are used, a detailed description of the sinker must be provided in the written procedure. It may be useful to evaluate different sinker types, recognizing that sinkers can significantly influence the dissolution profile of a dosage unit. When transferring the procedure, the same sinkers should be used, or if a different design is used, it should be shown to produce equivalent results. There are several types of commercially available sinkers. In (711), a harmonized sinker is described in Figure 2a.

A standard sinker can be made by using the appropriate length of wire and coiling it around a cylinder. For materials, use 316 stainless steel wire, typically 0.032 inch/20 gauge, or other inert material and wind the wire around cylinders of appropriate diameter (e.g., cork borers) for an appropriate number of turns to fit the capsule shell type. Sizes are shown in Table 2. The ends of the coil can be curved to retain the capsule within the sinker when they are immersed. Because the ends of the wire may be rough, they may need to be filed. If the sinker is handmade, the sinker material and construction procedure instructions should be documented (e.g., dimension, design, number of coils); if a commercial sinker is used, the vendor part number should be reported if available.

### Table 2. Wire Sinkers Used With Common Capsule Shell Sizes

<table>
<thead>
<tr>
<th>Capsule Shell Size</th>
<th>Length of Wire (cm)</th>
<th>Diameter Size (cm)</th>
<th>Cork Bore Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>#0, elongated</td>
<td>12</td>
<td>0.8</td>
<td>4</td>
</tr>
<tr>
<td>#1 and #2</td>
<td>10</td>
<td>0.7</td>
<td>3</td>
</tr>
<tr>
<td>#3 and #4</td>
<td>8</td>
<td>0.55</td>
<td>2</td>
</tr>
</tbody>
</table>

Although sinkers are typically used to keep the dosage form at the bottom of the vessel, they can also be used to keep dosage forms from sticking to the vessel (e.g., film-coated tablets). The sinker should be appropriate to the dosage form; therefore, the same sinker size may not be suitable for all dosage-form sizes. The sinker should not be too tight around the dosage form because this may restrict interaction with the medium. Conversely, if wrapped too loosely, the dosage form may escape soon after the test begins. The sinker should be small enough that the capsule does not change its orientation within the sinker. Care should be taken when testing capsules that have some cross-linking present, to keep the sticky shell from attaching to the vessel bottom. In this case, the harmonized sinker design provided in Figure 2a of (711) will be advantageous.

2.3 Agitation

For immediate-release capsule or tablet formulations, Apparatus 1 (baskets) at 50–100 rpm or Apparatus 2 (paddles) at 50 or 75 rpm are used commonly. Other agitation speeds are acceptable with appropriate justification. Rates outside 25–150 rpm for both the paddle and the basket are usually not appropriate because of mixing inconsistencies that can be generated by stirring too slow or too fast. Agitation rates between 25 and 50 rpm are generally acceptable for suspensions.

For dosage forms that exhibit coning (mounding) under the paddle at 50 rpm, the coning can be reduced by increasing the paddle speed to 75 rpm, thus reducing the artifact and improving the data. If justified, 100 rpm may be used with Apparatus 2, especially for extended-release products. Decreasing or increasing the apparatus rotation speed may be justified if to achieve an in-vitro–in-vivo correlation (IVIVC) the resulting profiles better reflect in vivo performance, or if the method results in better discrimination without adversely affecting method variability.

Apparatus 3 (reciprocating cylinder) can be used at dip rates ranging from 5 to 30 dips/min. The hydrodynamics are influenced by the cylinder’s reciprocating motion and the resulting movement of the sample in the medium. The reciprocating motion of the cylinder and screen may cause foaming if the medium contains surfactants. Addition of an anti-foaming agent such as simethicone or n-octanol may be useful for avoiding foaming from surfactants.

Apparatus 4 (flow-through cell) is described in (711) with standard flow rates of 4, 8, and 16 mL/min. Other flow rates for Apparatus 4 can be used if justified and if within the capacity of the pump to conform with the requirements in (711). Agitation in Apparatus 4 is not only related to the pump speed but can also be affected by cell diameter. At a set flow rate, as measured by volume, the 12-mm cell will develop a greater linear fluid velocity than is achieved in the 22.6-mm cell. Apparatus 4 can be configured with the addition of glass beads in the entry cone of the flow-through cell (packed column) or without glass beads (open column).

The flow characteristics of the flow-through cell are discussed in the scientific literature (15). The placement of the sample in the flow-through cell will influence the flow patterns that occur and thus should be a consideration in the attempt to reduce variability of the results.
2.4 Study Design

Selection of the agitation rate and other study design elements for the dosage form, whether immediate release or modified release, should conform to the requirements and specifications (i.e., apparatus, procedures, and interpretation) given in (711).

2.4.1 TIME POINTS

For immediate-release dosage forms, the duration of the dissolution procedure is typically 30–60 min; in most cases, a single time point specification is adequate for pharmacopeial purposes. For method development, however, a sufficient number of time points should be selected to adequately characterize the ascending and plateau phases of the dissolution curve. Industrial and regulatory concepts of product comparability and performance may require additional time points, which may also be required for product registration or approval. According to the Biopharmaceutics Classification System referred to in several FDA Guidances, highly soluble, highly permeable drugs formulated into very rapidly dissolving products need not be subjected to a profile comparison if they can be shown to release 85% or more of the drug substance within 15 min. For these types of products, a one-point test or disintegration will suffice. However, most products do not fall into this category. Dissolution profiles of immediate-release products typically show a gradual increase reaching 85%–100% at about 30–45 min. Thus, sufficient dissolution time points are chosen to characterize the performance for most immediate-release products. For some products, including suspensions, useful information may be obtained from earlier points, e.g., 5–10 min. For slower-dissolving products, time points later than 60 min may be useful. Dissolution test times for compendial tests are usually established on the basis of an evaluation of the dissolution profile data.

The $f_2$ similarity factor may not be useful when more than 85% is dissolved at 15 min. If the $f_2$ similarity factor is to be used, multiple time points for the dissolution test are required, with at least two time points with mean percent dissolved (typically for $n = 12$) below 85% dissolved and only one point above 85% for both products (16). Therefore, the addition of early time points may be useful.

For testing an extended-release dosage form, at least three time points are chosen, to guard against dose dumping, to define the in vitro release profile, and to show that essentially complete release (>80%) of the drug is achieved. Additional sampling times may be useful. Certain IVIVC criteria, such as level B correlation (according to In Vitro and In Vivo Evaluation of Dosage Forms (1088)), require the experimental determination of the time to dissolve 100% of the label claim. Selection of the final time points is reflective of the data from the drug release profile that are generated during development. For products containing more than a single active ingredient, determine the drug release for each active ingredient.

Delayed-release dosage forms usually require specifications for at least two time points; therefore, it is important during development to evaluate the entire dissolution profile. In the case of enteric-coated dosage forms, the functionality of the coating is usually proven by challenge in an acid medium, followed by a demonstration of dissolution in a higher-pH medium.

Chapter (711) gives a standard buffer medium for that stage of testing but other media may be used if justified. The timing of the acid stage is typically 2 h, and release in the buffer is similar to the timing for immediate-release forms. For delayed-release dosage forms that are not enteric coated, setting of specifications is different. Unlike delayed release, the onset of release is not determined by the experimental design, which is the pH change; multivariate specifications, therefore, may be needed to define time ranges and corresponding percentage ranges.

So-called infinity points can be useful during development studies. To obtain an infinity point, the paddle or basket speed is increased at the end of the run (after the last time point) for a sustained period (typically, 15–60 min), after which time an additional sample is taken. Although there is no requirement for 100% dissolution in the profile, the infinity point can be compared to content uniformity data and may provide useful information about formulation characteristics during initial development or about method bias.

2.4.2 OBSERVATIONS

Visual observations and recordings of product dissolution and disintegration behavior are useful because dissolution and disintegration patterns can be indicative of variables in the formulation or manufacturing process. For visual observation, proper lighting (with appropriate consideration of photo-degradation) of the vessel contents and clear visibility in the bath are essential. Documenting observations by drawing sketches and taking photographs or videos can be instructive and helpful for those who are not able to observe the real-time dissolution test. Observations are especially useful during method development and formulation optimization. It is important to record observations of all six vessels to determine if the observation is seen in all six vessels, or just a few. If the test is performed to assist with formulation development, provide any unique observations to the formulator. Examples of typical observations include, but are not limited to, the following:

1. Uneven distribution of particles throughout the vessel. This can occur when particles cling to the sides of the vessel, when there is coning or mounding directly under the apparatus (e.g., below the basket or paddle), when particles float at the surface of the medium, when film-coated tablets stick to the vessel, and/or when off-center mounds are formed.
2. Air bubbles on the inside of the vessel or on the apparatus or dosage unit. Sheen on the apparatus is also a sign of air bubbles. This observation would typically be made when assessing the need to deaerate the medium.
3. Dancing or spinning of the dosage unit, or the dosage unit being hit by the paddle.
4. Adhesion of particles to the paddle or the inside of the basket, which may be observed upon removal of the stirring device at the end of the run.
5. Pellicles or analogous formations, such as transparent sacs or rubbery, swollen masses surrounding the capsule contents.
6. Presence of large floating particles or chunks of the dosage unit, especially at the surface of the media.
7. Observation of the disintegration rate (e.g., percentage reduction in size of the dosage unit within a certain time frame).
8. Complex disintegration of the coating of modified or enteric-coated products, [e.g., the partial opening and splitting apart (similar to a clamshell) or incomplete opening of the shell], accompanied by the release of air bubbles and excipients.
9. Whether the dosage form lands in the vessel center or off-center, and if off-center, whether it sticks there.
10. Time required for the complete dissolution of the capsule shell or for tablet disintegration.

Observations also help to document that the proper procedure has been followed, or more importantly, that a deviation has occurred. Examples include the confirmation that a dosage form is actually in the vessel during the test or that more than one dosage form are inadvertently in the same vessel, or that a filter from the autosampler has dropped into the vessel.

2.4.3 SAMPLING

Manual: For manual sampling, use chemically inert devices (e.g., polymeric or glass syringes, and polymeric or stainless steel cannula), a filter, and/or a filter holder. The sampling site must conform to specifications in 711. When the agitation conditions are very slow, e.g., a 50-rpm basket, care should be taken to sample consistently in the same location in the vessel because there may be a concentration gradient; avoid sampling very close to the shaft or vessel wall. During method development, a decision should be made regarding whether to replace the media after each time point. Replacement is not preferred because the dosage unit may be disturbed during delivery of the media. However, replacement may be necessary if maintaining sink conditions is a challenge. With replacement, the volume used in the calculations remains the same throughout the time points, but there is some drug substance withdrawn with each sample that will need to be accounted for in the calculations.

Metal surfaces may interact with the sample. For example, adsorption onto metal surfaces may occur, or the metal surfaces may release metal ions into aqueous media. The ions can then catalyze degradation reactions, leading to artifacts during the analytical procedures. The surfaces of stirring elements and metal locks of syringes may be sources of interference to accurate sampling.

Autosampling: Autosampling is discussed in section 4. Automation.

2.4.4 CLEANING

Importance is placed on evaluation of the cleaning process between tests. Changes of dissolution medium and/or product necessitate the need for cleaning. Residues on the vessels can affect the results (e.g., adsorbed residues may dissolve and alter subsequent media properties or interfere with the sample analysis), and effective cleaning will return them to a suitable state. Automated systems are discussed in section 4.4 Cleaning.

Change to read:

2.5 Data Handling

Dissolution rates are calculated from the change in drug concentration in the dissolution medium. For procedures in which the volume of medium is fixed, such as for Apparatus 1 and Apparatus 2 testing of immediate-release dosage forms with only one sampling time, the concentration of the sample is multiplied by the medium volume to arrive at the mass of drug dissolved usually expressed as percentage of label claim. When multiple time points are taken, the total amount of drug removed at earlier time points should be assessed and may be part of the calculation of the amount dissolved, if considered important. Similarly, if the medium volume is not fixed, for example when the sample volume is not replaced in testing extended-release products, the change in medium volume must be part of the calculation for successive sampling points. Dissolution tests performed with Apparatus 4 in the closed-loop configuration with in situ detection provide a convenient control of the medium volume. For testing with Apparatus 4 in the open configuration, the test time and flow rate will determine the volume of medium used in the dissolution calculations.

Dissolution results can be evaluated as either cumulative rates or fractional rates. Cumulative rates represent the sum of all drug dissolution that occurs during an interval (Figure 1). Fractional rates are assessed at a specific time point or during a portion of the total test time (Figure 2). Typically, the rate of release will be expressed as either mass or percentage of label claim per unit time. For most compendial dissolution testing, the dissolution rate is expressed as a percentage of the label claim dissolved at the indicated test time.

![Figure 1](https://example.com)  
**Figure 1.** An example of a plot of dissolution as a cumulative process. Concentration, C, is the amount of drug released per volume of medium, and t represents time. This type of plot is readily observed in constant-volume dissolution systems, such as Apparatus 1 or Apparatus 2, or Apparatus 4 in closed-loop configuration.
Figure 2. An example of a plot of the observed concentration of the sample taken for an interval that is negligibly small in relation to the time of the overall dissolution process. This concentration is proportional to the instantaneous or fractional dissolution rate \( \frac{dc}{dt} \). This type of plot is readily observed in continuous-flow dissolution systems, such as Apparatus 4 in open-loop configuration.

Cumulative dissolution profiles represent the total amount of drug dissolved from the formulation over time. When cumulative dissolution is measured in a constant-volume system, no correction for the amount lost in sampling needs to be made. If sample is removed from the system, the amount consumed in analysis must be accounted for in the calculation. Recirculated sampling with Apparatus 1 or Apparatus 2, or with Apparatus 4 in the closed-loop configuration (Figure 3), are all examples of systems that will produce cumulative dissolution rates. With Apparatus 4 in the open configuration (Figure 4), cumulative rates accounting for the total amount of drug dissolved across the testing interval are obtained by collecting and analyzing the entire outflow from each individual flow-through cell. With Apparatus 3 (Figure 5), the medium in each tube is sampled at the end of the programmed interval, and the analyzed concentration represents the cumulative dissolution rate during that interval.

Figure 3. Apparatus 4 in the closed-loop configuration.

Figure 4. Apparatus 4 in the open-loop configuration. The sample can be collected in fractions, as shown at the top. The medium can be changed by using successive reservoirs.

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Fractional dissolution rates are typically measured for a discrete interval. A series of such rates will produce a step function as the dissolution profile. At any time, the cumulative dissolution rate from this type of profile is the sum of the preceding intervals. This type of profile is represented by Apparatus 3 using multiple tubes or Apparatus 4 in the open-loop configuration where the total outflow is collected and analyzed for successive intervals.

A number of algebraic and numerical methods exist for transforming cumulative and fractional dissolution results. The difference in amount released for successive time points can be calculated, and the average release rate is determined by the formula:

\[
\text{Result} = \frac{(M_2 - M_1)}{(t_2 - t_1)}
\]

\(M\) = mass or percentage of label claim
\(t\) = time

As the difference of \(t_2\) from \(t_1\) is reduced, the average rate can be considered to approach an instantaneous rate. Sampling considerations and physical constraints on measurement of the mass transfer at the medium interface of the dosage form make the measurement of true instantaneous dissolution impractical for routine determination in the laboratory. Fractional dissolution is measured for intervals where the difference between \(t_2\) and \(t_1\) is small, relative to the total test time. The design of Apparatus 4 in the open configuration permits a direct measurement of the fractional dissolution over small time intervals. For example, if a 4-mL fraction of outflow for Apparatus 4 running 16 mL/min is sampled, either by in situ detection or offline, the amount of drug detected represents the dissolution occurring in a 15-s interval.

Pooled dissolution has been used in a number of monographs. The pooled dissolution procedure produces an average release rate for the units tested by combining equal volumes from each vessel or cell and performing analysis of only the one resulting solution. Because this approach uses only the average release rate for comparison with the acceptance table, the pooled dissolution procedure has been viewed as reducing the amount of data available from the dissolution test and, thus, reducing its value. However, it should be noted that the pooling of equal sample volumes is equivalent, from a calculation standpoint, to determining the arithmetic mean of the individual sample results.

For the purpose of correlation with in vivo data, parameters of mathematical models are obtained by fitting to dissolution data to establish a continuous functional relationship called IVIVC (see (1088)).

2.6 Dissolution Procedure Assessment

The dissolution procedure requires an apparatus, a dissolution medium, and test conditions that together provide a method that is sensitive to changes in critical quality attributes, yet sufficiently rugged and reproducible for day-to-day operation. The method should be able to be transferred between laboratories.

The ideal dissolution procedure will not contribute an unacceptable degree of variability and will provide a profile with adequate points below 85% dissolved. If 85% dissolved occurs before 15 min, then \(f_2\) comparisons may not be appropriate.

There are many ways to challenge the sensitivity of the method. One option is to compare dissolution profiles of formulations that are intentionally manufactured with meaningful variations for the most relevant critical manufacturing variable, for example, ±10%–20% change to the ranges of these variables. Similarly, samples that have been stressed may be used to demonstrate sensitivity to changes on stability. This concept may be used to establish the factors that are most significant in their influence on the dissolution rate. These studies can focus on either the dissolution parameters (e.g., media concentration, agitation rate, and deaeration) or the product attributes (e.g., excipient ratios, particle size, compression). The ultimate goal is to understand the release mechanisms and determine whether the dissolution procedure can show change in the critical quality attributes of a drug product.
3. ANALYTICAL FINISH

The dissolution step has been described as an involved sample preparation. The sample handling and analytical procedure that are used to determine the amount of drug substance dissolved during the dissolution procedure are termed the “analytical finish.” Although spectrophotometric determinations and HPLC are used most commonly and are discussed in this chapter, any suitable analytical technology may be used. Section 5. Validation describes criteria for the methods.

3.1 Sample Processing

After the samples are withdrawn from the dissolution medium, they may require additional processing to make them suitable for the analytical methodology used to determine the amount released. For example, filtration may be used to remove undissolved particulate matter, or samples may need to be protected from exposure to light or may need refrigerated storage. In addition, samples may have to be diluted to a level that is within the linear range of the method. With analysis by HPLC, dilution of the sample with mobile phase may be necessary to reduce the effect on the separation of injecting dissolution medium. Other types of treatment may be necessary depending on the product formulation, such as the inactivation or elimination of interference caused by components of the formulation by the addition of appropriate reagents. However, separation may not be possible or needed in all cases. In some cases, in situ measurements obtained with methods such as fiber optics or electrochemical determination may be useful.

3.2 Filters

The topic of filtration is discussed in section 1.1 Performing Filter Compatibility.

3.3 Centrifugation

Centrifugation of samples is not preferred, for several reasons: dissolution can continue to occur until the solids are removed, a concentration gradient may form in the supernatant, and energy imparted may lead to increased dissolution of the drug substance particles. Possible exceptions, when centrifugation could be preferred, might include the use with compounds that adsorb onto all common filters, or situations when the potential filter leachables and extractables might interfere in the quantitative step of the dissolution test (e.g., when fluorescence procedures are used in quantitation). Centrifugation may prove useful during method development for evaluating the suitability of the filter material.

3.4 Analytical Procedure

The usual assay for a dissolution sample employs either a spectrophotometric procedure or a liquid chromatographic procedure. Spectrophotometric determination may be direct or may provide the detection for HPLC. Spectrophotometric determination is used often because results can be obtained faster, the analysis is simpler, it is easier to automate, and fewer solvents are needed. The use of direct spectrophotometric determination typically requires confirmation of specificity. HPLC is preferred for a number of reasons such as providing a wide dynamic range that reduces the need to dilute some samples while also providing sensitivity in the analysis of dilute samples, and greater selectivity when excipients or multiple drugs in the formulation present a significant interference. Modern HPLC systems employ autosamplers that provide speed and simplicity advantages comparable to spectrophotometric analysis.

3.5 Spectrophotometric Analysis

Direct spectrophotometric analysis may be performed on samples that are manually introduced to the cuvette. Alternatively, samples may be automatically introduced into the spectrophotometer using autosippers and flow cells. Routine performance checks, cleaning, and maintenance, as described in the standard operating procedures or metrology documents, help to ensure reliable operation of these instruments. Cells with path lengths ranging from 0.02 cm to 1 cm are typically used, and longer path-length cuvettes can be used to increase the range for quantification of dilute samples. Cell alignment and air bubbles could be sources of error. The shorter path-length cells are used to avoid diluting the sample; in all cases, however, acceptable linearity and standard error need to be demonstrated.

The choice of wavelength for the determination should be based on the spectrum of the drug in solution. In some cases, where the drug substance can degrade in the dissolution medium (e.g., dosage forms containing aspirin), it is useful to carry out the measurements at the isosbestic point. Excipients can also have effects, but performing analysis at multiple wavelengths can minimize their effects. The contribution of the absorbance from an excipient at the analytical wavelength can sometimes be determined by ratio from its absorbance at a wavelength where the absorbance of the drug substance is minimal.

Using a validated analytical finish, standard solutions are typically prepared in dissolution media and analyzed at just one concentration, either at 100% of the dosage strength or the selected Q value because linearity of the analytical finish has been established. Prior to validation, dissolution profile analysis, or analysis of products of various strengths, requires using multiple standard solutions covering the expected range of concentration. A typical media blank, standard, and sample may be analyzed in a sequence that brackets the sample with standards and blanks, especially at the beginning and end of the analysis. The standard and sample solutions should both be prepared in the dissolution medium in the linear concentration range and measured at the same wavelength. However, small amounts of an organic solvent may be used in the preparation of the standard, provided that the accuracy criteria can be met during validation.

The absorptivity is calculated by dividing the mean standard absorbance by the concentration, in mg/mL, divided by the cell path length in cm. A rearrangement of the Beer-Lambert expression gives the absorptivity, \(a\), as:
This value may be useful in troubleshooting aberrant data. Fiber optics as a sampling and determinative method, with proper validation, are an option.

### 3.6 HPLC

For HPLC analysis, the effect on the chromatogram of peaks resulting from injection of dissolution media require enumeration. A large solvent disturbance may affect accuracy and precision of response if it is poorly resolved from the peak of interest. This is even more important if large injector volumes (>100 µL) are needed. System suitability tests may evaluate peak shape; separation of the main peak from solvent disturbance and from closely eluting peaks; and injection precision. At a minimum, the precision is critical.

Ideally, the standard solutions should be diluted with the dissolution media at a concentration within the linear range of the method, e.g., 100%, or the selected Q value of the dosage strength. However, organic solvent may be used in the preparation of the standard, provided that the accuracy criteria can be met during validation. In some cases, the sample may be diluted with mobile phase to improve the peak shape. The standard and sample solutions should both be prepared in the linear concentration range and measured at the same wavelength.

### 4. AUTOMATION

Automated dissolution systems may be configured in various ways and degrees. The elements of test preparation, initiation, sampling and timing, and cleaning all can be automated. Fully automated systems are available, as are systems where individual steps, such as media preparation or sampling, are automated. This section will discuss operational steps that can be automated. The level of complexity for automation depends on whether the instrument configuration is open or closed loop and also whether the analytical device is coupled online or offline. Online analysis returns the sample aliquot to the test system, as in the case of spectrophotometry with flow-through cuvettes. Offline analysis removes the sample aliquot from the dissolution medium for subsequent analysis, typically by HPLC, where the analysis consumes the sample. The decision on the configuration usually depends on the number of samples to be processed and the time required for their analysis.

Operational steps that are not part of the compendial procedure should be validated. Deviations from the standard procedure described in (711), such as use of sampling probes or fiber-optic probes, should be validated against the standard procedure.

#### 4.1 Medium Preparation

Automated media preparation generally is accomplished by diluting concentrates. Automated media preparation systems typically dispense the volume of medium into the vessel by monitoring either the weight or volume. Chemical and physical stability of the concentrates as well as homogeneity of the dilutions over the intended period of use are important issues and should be understood. Concentrates of buffer solutions and surfactants may have stability issues, such as chemical degradation and pH change. Physical instability may manifest as precipitation, re-crystallization, or phase separation and should be prevented.

If deaeration of the medium is required, the level of deaeration should be specified. The concentration of the dissolved oxygen can be used to evaluate the efficiency of deaeration procedures discussed in section 2.1 Deaeration.

#### 4.2 Sample Introduction and Timing

Samples should be inserted in the vessel in a reproducible way. Automated sample introduction and aliquot withdrawal provide an advantage over manual sampling because the automated techniques can reduce the variability in the vessel-to-vessel timing of the test intervals. However, automated sample handling may impose timing limitations that need to be considered. The pharmacopeial tolerance of ±2% of the specified dissolution test time may be difficult to meet for early time points.

#### 4.3 Sampling and Filtration

Autosampling is a useful alternative to manual sampling, especially if the test includes several time points. The transfer and filtration of sample solutions from the dissolution instrument to the analytical unit may be undertaken via tube connections or via robotic devices operated in a stepwise procedure. Sample volumes may be removed from the dissolution medium and not returned (consumptive sampling), or the sample volume may be returned to the dissolution medium (recirculated sampling).

There are many brands of autosamplers, including semi-automated and fully automated systems. Routine performance checks, cleaning, and maintenance, as described in the pertinent standard operating procedures or metrology documents, help to ensure reliable operation of these devices.
Sampling probes may or may not remain in the vessel throughout the entire run. Sampling probes or fiber-optic probes can disturb the hydrodynamics of the vessel; therefore, adequate validation should be performed to ensure that the probes are not causing a significant change in the dissolution rate. If filters are used that are different from those used for manual sampling, then these different filters should also be evaluated separately. The position of the pharmacopeial sampling zone for Apparatus 1 and Apparatus 2 is midway from the top of the stirring element to the medium surface and depends on the medium volume. Sampling probes should pull the sample from the sampling zone. Instruments for which the sampling occurs through the hollow shaft should be designed with a means to adjust the depth of the inlet aperture to allow conformance with this requirement. The programmed sampling volume depends on the dead volume of the tubing, cuvettes, and other devices and has to be adjusted accordingly.

A recirculated sampling alignment can be operated either by discharging the tubing contents into the vessel after each sampling or by allowing the tubing to remain filled with solution in the intervals between sampling points. In the latter case, the dead volume and carryover effects are important considerations.

The need for sample volume replacement should be considered. In consumptive sampling with multiple sampling time points, the withdrawn volume may be replaced with an equal volume of fresh medium. The sampling volume may be critical if, in total, it exceeds 1% of the stated volume of dissolution medium required by the procedure. If it can be shown that replacement of the medium is not necessary, the volume change must be part of the calculation of results. See section 2.5 Data Handling.

Carryover may occur when subsequent samples are affected by residues or conditions of previous samples; the effect of the first sample or condition “carries over” to the second. In liquid handling, residues of liquids previously in the sample solution may contaminate subsequent sample solutions. Dissolution media containing surfactants or lipids may present problems.

Carryover may occur for successive samples taken over a multiple time-point test, as well as at the beginning of a new test due to the cleaning solution. This topic is discussed in section 4.4 Cleaning.

Interaction of dissolved drug substance with the sampling and transfer devices is an important consideration. When adsorption of the dissolved drug substance occurs, it most often involves surfaces of the dissolution apparatus or sampling filters and tubing. Adsorption may be pH dependent in the case of charged, dissolved drug substance. Adsorption of the dissolved drug to the parts of the sampling device should be assessed using a typical sample solution (dissolution sample from the product or drug substance with formulation matrix) with known concentration. The typical design is a cross-validation with aliquots of the same sample solution passing and bypassing the sampling device (including the sampling probe, filter, tubing, valves, and pump). There is no general recommendation that may give preference to any kind of material or equipment construction (e.g., glass or specific polymers). See section 5.7 Considerations for Automation for more information.

In addition to the information in section 2.4.3 Sampling, connections of pumps and tubing may be sources of contamination in automated systems. Interferences with the spectroscopic analytical procedures, which are commonly used for dissolution testing, are less of a concern. However, interferences must be evaluated if the product under investigation contains low-dose metal salts, as do some dietary supplements.

Liquid transfer usually is undertaken via polymeric tubing. Inert materials such as polytetrafluoroethylene (PTFE) sometimes cannot be used because of their mechanical properties. Where flexible tubes are required, for example in peristaltic pumps or for coiling in a small radius, polypropylene (PP) or high-density polyethylene (HDPE) may be the preferred materials. Depending on the type of polymer and its crystallinity and density, leaching of constituents, mainly plasticizers, may occur. Leachables can interfere with the analytical procedure. The concentration leached to the sample solution usually depends on the surface, the temperature, the exposure time, the hydrodynamic conditions, and the composition of the media.

### 4.4 Cleaning

In addition to the information in section 2.4.4 Cleaning, automated systems have specific cleaning issues. For example, evaluation of the effectiveness of purging and rinsing between sampling times and within-run condition of the tubing is recommended. Also it is important to evaluate the cleaning process between tests.

### 4.5 Operating Software and Computation of Results

The software systems for data evaluation and instrument operation must be validated as per 21 CFR 11 (17).

### 4.6 Common Deviations from the Compendial Procedures That May Require Validation

Some common areas of deviation from compendial procedures include the following:

- Sample introduction relative to start of spindle rotation
- Residence time and positioning of sampling probes
- Recirculated versus consumptive sampling
- Sample volume replacement in consumptive sampling.

### 5. VALIDATION

The validation topics described in this section are typical but not all-inclusive and can be viewed in the context of Validation of Compendial Procedures (1225), as well as the International Conference on Harmonization (ICH) document, Validation of Analytical Procedures (18). Validation for both parts of the dissolution procedure, the analytical finish and the dissolution step, will be discussed in this section. The dissolution step is the release of the drug in the dissolution medium and sampling. The analytical finish is defined in section 3. Analytical Finish. Validation of the analytical finish will evaluate the attributes, linearity and range, precision, specificity, accuracy/recovery, robustness, and stability of the sample and standard solutions. Validation
of the dissolution step will include evaluation of precision and robustness of the dissolution sample preparation. Validation of the analytical finish is performed either using a standard solution or spiked placebo or by the method of standard addition (spiked drug product as described in Accuracy in \(1225\)), as specified in the sections below. Validation of the dissolution step requires the use of a well-characterized dosage form (e.g., having tight content uniformity and uniform performance). Depending on the parameter of interest, validation of the sample handling and analytical procedure can be performed in situ, e.g., within the dissolution vessel. The validation parameters addressed and the extent of the validation may vary, depending on the phase of development or the intended use for the data.

The acceptance criteria are presented as guidelines only, and may differ for some products. Manufacturers should document the appropriate acceptance criteria for their products in pertinent Standard Operating Procedures (SOPs) or in validation protocols. Other considerations may be important for special dosage forms. Validation studies should be performed across the range of profile time points. For products containing more than a single active ingredient, the dissolution procedure needs to be validated for each active ingredient. It is expected that investigations into filter suitability and the potential for glass adsorption will have been undertaken already (see 1.1 Performing Filter Compatibility). Validation of these assessments may occur during spiked recovery experiments.

### 5.1 Specificity/Placebo Interference

It is necessary to demonstrate that the results are not unduly affected by placebo constituents, other active drugs, or degradants. The placebo consists of all the excipients and coatings, with inks and capsule shells included if appropriate, without the active ingredient. Placebo interference can be evaluated by using a spiked placebo that is prepared by weighing samples of the placebo blend, dissolving or dispersing them in dissolution medium at concentrations that would be encountered during testing, and adding a known amount of the drug in solution. It may be preferable to perform this experiment at 37°, comparing the solution to a standard solution at the concentration expected to be encountered during testing, by using the formula:

\[
\text{Result} = \left( \frac{A_p - A_s}{A_s} \right) \times C_s \times \left( \frac{V}{L} \right) \times 100
\]

- \( A_p \) = absorbance of the placebo
- \( A_s \) = absorbance of the standard
- \( C_s \) = concentration of the standard (mg/mL)
- \( V \) = volume of the medium (mL)
- \( L \) = label claim (mg)

The interference should not exceed 2%. Note that for extended-release products, a placebo version of the finished dosage form may be more appropriate than blends because this placebo formulation will release the various excipients in a manner more nearly reflecting the product than will a simple blend of the excipients. In this case, it may be appropriate to evaluate potential interference at multiple sampling points in the release profile, with worst-case interference expected at the later sampling points.

The blank is the dissolution medium without dissolved sample, and it is treated in the same manner as the sample. The effect of the absorbance of the blank at the analytical wavelength should be evaluated. In most cases, the absorbance of the dissolution medium blank may not exceed 1% of the standard solution at the concentration used for analysis. Values >1% should be evaluated on a case-by-case basis. If the placebo interference exceeds 2%, modification of the method may be necessary. Possible modifications include choosing another wavelength, subtracting baseline using a longer wavelength, transforming absorbance values (e.g., first derivative), and using an alternative analytical technique such as HPLC. Other means for minimizing the placebo interference would be acceptable with appropriate justification. When other active drug substances or significant levels of degradants are present, it is necessary to show that these do not significantly affect the results. One procedure for doing this is to measure the matrix in the presence and absence of the other active drug substance or degradant: any interference should not exceed 2%. Similar approaches may be used if other techniques are used for the analytical finish.

### 5.2 Linearity and Range

Linearity is typically established by preparing solutions of the drug substance, ranging in concentration from less than the lowest expected concentration to more than the highest concentration during release. The solutions may be prepared either using either a standard solution or spiked solution or by the method of standard addition. A minimum of five concentrations is normally used (see \(1225\)). Typically, solutions are made from a common stock if possible. The concentration range may not exceed the linearity limits of the method, including the instrumentation. Organic solvents may be used to enhance drug solubility for the preparation of the linearity standard solutions. However, no more than 5% (v/v) of organic solvent should be present in the final solution unless validated. Linearity is typically calculated by using an appropriate least-squares regression program. Typically, a square of the correlation coefficient \( r^2 \geq 0.98 \) demonstrates linearity. In addition, the \( y \)-intercept must not be importantly different from zero.

The range of the procedure is the interval between the upper and lower concentrations of the drug substance (including these levels) that has been demonstrated to have a suitable level of precision, accuracy, and linearity using the procedure as written.

### 5.3 Accuracy/Recovery

Accuracy/recovery is typically established by preparing multiple samples containing the drug substance and any other constituents present in the dosage form (e.g., excipients, coating materials, capsule shell) ranging in concentration from less
than the lowest expected concentration to more than the highest concentration during release. Accuracy/recovery may be done in conjunction with linearity determination. The method of standard addition can also be used. Before this activity, it is expected that filter assessment will already have been performed, and adsorption of drug onto the glass has also been investigated and ruled out.

Individual solutions may be directly prepared in the dissolution medium. Alternatively, to enhance drug solubility it may be appropriate to prepare a stock solution by dissolving the drug substance in a small amount of organic solvent (typically not exceeding 5% organic solvent in the final dissolution media) and diluting to the final concentration with dissolution medium. An amount of stock solution equivalent to the targeted label claim may be used instead of the drug substance powder. Similarly, for very low strengths, it may be more appropriate to prepare a stock solution than to attempt to weigh very small amounts.

The measured recovery is typically 95%–105% of the amount added. Bracketing or matrixing of multiple strengths may be useful. A special case for validation is the Acid Stage procedure described in (711), Delayed-Release Dosage Forms. The limit of NMT 10% needs to be validated. Recovery experiments for drugs that have low solubility in acidic media may be challenging or impossible to perform and may need to be addressed on a case-by-case basis. If the compound degrades in acid, the validation experiment must address this fact.

5.4 Precision

5.4.1 REPEATABILITY OF ANALYSIS

For the analytical finish, repeatability is evaluated by obtaining replicate measurements of standard and/or spiked placebo/standard addition solutions. It can be determined by calculating the RSD of the multiple injections or spectrophotometric readings for each standard solution, or by using the accuracy or linearity data. ICH guidance, Validation of Analytical Procedures: Methodology, recommends that repeatability should be assessed using a minimum of nine determinations covering the specified range for the procedure (i.e., three concentrations and three replicates of each concentration) or using a minimum of six determinations at 100% of the test concentration. A typical acceptance criterion is an RSD of <2%. The demonstration of the repeatability for the dissolution step is conducted by performing the dissolution step on separate units of a well-characterized dosage form or equivalent composite.

5.4.2 INTERMEDIATE PRECISION/RUGGEDNESS

Assuming that the major contributor to the variance is from the dissolution step, intermediate precision may be evaluated to determine the effects of random events on the precision of the dissolution procedure. This evaluation is typically done later in the development of the drug product and is required for full method validation. For many analytical procedures intermediate precision is typically assessed by determination of contributions to variance and, possibly, by a comparison of means. The use of an experimental matrix design is encouraged for evaluation of intermediate precision because interaction effects may be observed more clearly relative to a single variable experiment. In dissolution testing, a ruggedness approach that compares means alone is often taken to investigate the factors that contribute to intermediate precision. The ruggedness can be evaluated across the range of product strengths. Typical variations to be studied include different days, analysts, and equipment. If possible, ruggedness can be evaluated using a drug product lot if well characterized, for example, by having tight content uniformity and uniform performance, but if this type of lot is not available, a premeasured placebo with active ingredients may be used to investigate the intermediate precision. The use of such a spiked placebo would additionally support the assessment of the contribution of the analytical finish to the observed variability of results.

The dissolution procedure on the same lot of well-characterized dosage form may be run by at least two different analysts from the same laboratory, with each analyst preparing the standard solutions and the medium and following the defined extraction/quantification procedure. Typically, the analysts use different dissolution baths, spectrophotometers or HPLC equipment (including columns), and autosamplers, and they perform the test on different days. Full profiles are assessed where relevant to the product. This procedure may not be necessary at each strength; instead, bracketing with high and low strengths may be acceptable.

Acceptance criteria for intermediate precision or for ruggedness are predetermined. A typical acceptance criterion for ruggedness is that the difference in the mean value for dissolution results between any two conditions, using the same strength, does not exceed an absolute 10% at time points with <85% dissolved and does not exceed 5% for time points >85%. Acceptance criteria may be product specific and other statistical tests and limits may be used.

5.4.3 REPRODUCIBILITY

Reproducibility follows the general concepts of intermediate precision, but is performed by two different analysts at different labs.

5.5 Robustness

Evaluation of robustness, which assesses the effect of making small, deliberate changes to the dissolution conditions, typically is done later in development of the drug product and is a component for full method validation. It is performed using a well-characterized lot of drug product, for example having tight content uniformity and uniform performance. The number of replicates (typically 3 or 6) is dependent on the intermediate precision. All profile points should be evaluated.

Selection of parameters to be varied depends on the dissolution procedure and analysis type. The parameters may include medium composition (e.g., buffer or surfactant concentration, pH, deaeration), volume, agitation rate, sampling time, and temperature. Statistical analysis of the data generated will help determine the extent to which the parameters must be controlled in the method. The robustness assessment is well suited to Design of Experiments (DoE) methodologies to efficiently investigate the impact of the individual parameters and/or their interaction.
Robustness of analytical finish is referenced in (1225). HPLC analysis parameters may include mobile phase composition (percentage organic, buffer concentration, pH), flow rate, wavelength, column temperature, and multiple columns (of the same type). For spectrophotometric analysis, the wavelength may be varied.

5.6 Stability of Standard and Sample Solutions

The standard solution is stored under conditions that ensure stability. The stability of the standard solution is analyzed over a specified period of time (for at least the time of the entire dissolution procedure), using a freshly prepared standard solution at each time interval for comparison. The acceptable range for standard solution stability is influenced by the concentration and is typically between 98% and 102% at the expected final concentration.

The sample solution is typically stored at room temperature. The sample is analyzed over a specified period of time, using the original sample solution response for comparison. The typical acceptable range for sample solution stability may be between 98% and 102%, compared with the initial analysis of the sample solutions. If the solution is not stable, aspects to consider include temperature (refrigeration may be needed), light protection, and container material (plastic or glass).

The procedure may state that the standards and samples need to be analyzed within a time period demonstrating acceptable standard and sample solution stability.

5.7 Considerations for Automation

Automated methods offer opportunities for increased precision and reproducibility; however, bias may be introduced. In particular, the sampling probe and the sample lines warrant attention as places where inaccuracies may occur. Deviations from the procedure described in (711), such as resident sampling probes, sampling through the stirring element shaft (hollow-shaft sampling), or fiber-optic probes, should be validated. Other aspects of automation validation may include carryover of residual drug, effect of an in-residence probe, adsorption of drug, and cleaning and/or rinse cycles. Validation is performed using the automated dissolution system including materials. Therefore, any change in materials will require demonstration of suitability based on the validation attributes that are impacted by the change.

Manual and automated procedures should be compared to evaluate the interchangeability of the procedures. This is done by performing two automated runs at each dosage concentration, using all sampling points, compared to manually sampled runs of the same samples. The effect of the in-resident probe cannot be determined by sampling both ways from the same vessel. Results should be consistent with the requirements for intermediate precision if the procedures are to be considered interchangeable. The difference in the mean value for dissolution results between any two conditions using the same strength should not exceed an absolute 10% at time points with <85% dissolved nor exceed 5% for time points >85%. Acceptance criteria may be product specific, and other statistical tests and limits may be used.

Revalidation may be necessary when the automated system is used with different formulations because of the interaction with excipients. Dissolution media containing surfactants or lipids may require additional validation efforts.

6. ACCEPTANCE CRITERIA

The acceptance criteria should be consistent with historical release or stability data. There is an expectation that acceptable batches will have results that fall within the acceptance criteria and that all manufactured batches should have similar dissolution behavior, thus highlighting the importance of having a method that is not highly variable. The acceptance criteria and time point(s), therefore, should discriminate between an acceptable and an unacceptable batch. In addition, the dissolution test results are viewed as a link to the pivotal clinical trial batches. When changes in dissolution rate have been shown to affect bioavailability significantly, the dissolution test and acceptance criterion should distinguish batches with unacceptable bioavailability (19). Likewise, when changes in the formulation and manufacturing process significantly affect dissolution and such changes are not controlled by another aspect of the specification, the dissolution test and criteria should distinguish these changes.

6.1 Immediate-Release Dosage Forms

Although release and stability data are collected during dosage form development, it is common to record the entire dissolution profile or the amount of drug dissolved at specified intervals, such as 10, 20, 30, 40, 50, and 60 min or 15, 30, 45, and 60 min. At registration, dissolution for an immediate-release tablet usually becomes a single-point test. The acceptance criterion and test time are established by evaluating the dissolution profile data. The acceptance criterion for a dissolution test is a function of Q, which is expressed as a percentage of label claim of drug dissolved at a specified time. Typical Q values are in the range of 75%–80% dissolved. Q values in excess of 80% are not generally used because allowance needs to be made for assay and content uniformity ranges.

6.2 Delayed-Release Dosage Forms

The discussion about dissolution of delayed-release dosage forms in (711) focuses on enteric-coated dosage forms, which is the most common delayed-release dosage form. A dissolution test for a delayed-release tablet or capsule is a two-part test, and each part has acceptance criteria. First, the dosage forms are exposed to an acid medium, followed by exposure to a buffer medium. To ensure that the enteric coating performs properly, a “NMT” acceptance criterion is indicated in (711) for the acid stage. The medium used for an acid stage is usually 0.1 N HCl, and the duration of this stage is typically 2 h. The dosage forms are then exposed to a buffer medium, usually 0.05 M phosphate buffer at pH 6.8, but other buffers and pH targets may be used if justified. The duration of the buffer stage is usually 45 min for compendial tests, but this duration may vary, depending
on the drug product. As with immediate-release dosage forms, a Q value and time point are determined by evaluating the entire dissolution profile.

### 6.3 Extended-Release Dosage Forms

A dissolution test for an extended-release dosage form is generally similar to that used for an immediate- or delayed-release drug product, except that the duration of the test is longer, and at least three time points are specified for pharmacopeial purposes (20). Additional sampling times may be required for drug approval purposes. An early time point, usually 1–2 h, is chosen to show that dose dumping is not probable. An intermediate time point is chosen to define the in vitro release profile of the dosage form, and a final time point is chosen to show essentially complete release of the drug (20). The time points for the test should be determined by evaluating the dissolution profile across the desired test duration. Often, additional time points are obtained during dosage form development to aid with selecting the appropriate time points for the specification or monograph.

As with an immediate- or delayed-release drug product, the acceptance criteria and time points for an extended-release drug product should discriminate between an acceptable and an unacceptable batch. The acceptance criteria for the first stage of testing (L₁) should be established on the basis of available batch data (19,20). If human bioavailability data are available for formulations exhibiting different release rates, then an in vitro/in vivo relationship may be used to establish acceptance criteria (19,20). Acceptance criteria for the second (L₂) and third (L₃) stages are derived from the L₁ criteria using Acceptance Table 2 in (711).

### 6.4 Multiple Dissolution Tests

Typically, monographs for extended-release dosage forms contain multiple dissolution tests representing specific products. In accordance with General Notices, 4.10.10 Applicability of Test Procedures, the appropriate test, if not Test 1, is indicated on the product labeling. For example, the USP monograph for Oxycodone Hydrochloride Extended-Release Tablets (21) lists two dissolution tests, each of which has either three or four time points. If the Tablets are analyzed using Test 2 and the dissolution results comply with the criteria provided in the monograph, the labeling for Tablets can indicate that the Tablets meet USP Dissolution Test 2. Multiple dissolution tests also can be found in monographs for immediate- and delayed-release dosage forms. For example, the USP monographs for Levothyroxine Sodium Tablets and Pantoprazole Sodium Delayed-Release Tablets provide four dissolution tests (22,23).

### 6.5 Interpretation of Dissolution Results

The Interpretation section of (711) discusses immediate-, delayed-, and extended-release dosage forms. The discussion for each of these release patterns is expanded here with examples to assist with applying the criteria during the various stages of testing. Understanding how these criteria are applied will assist in setting appropriate acceptance criteria.

#### 6.5.1 IMMEDIATE-RELEASE DOSAGE FORMS

Once the Q value is established, the dissolution test is a staged test of three levels. In the first level of testing called S₁, six dosage forms are tested. Each dosage form must be Q ± 5% (absolute percentage points) dissolved at a specified time. For example, the time and tolerances in a monograph would be:

- **Time:** 30 min
- **Tolerances:** NLT 80% (Q) of the labeled amount of “drug substance” is dissolved.

If the Q value for a 200-mg label claim (LC) immediate-release tablet is specified as 80% and the time point is 30 min, then NLT 85% LC (170 mg) of the drug substance in each tablet must be dissolved at 30 min.

If this criterion is not met, then 6 additional tablets are tested at level 2 (S₂). To pass the S₂ acceptance criteria, the average of all 12 tablets must be equal to or greater than Q (80% LC; 160 mg in the above example), and no tablet has less than Q – 15% (65% LC; 130 mg in the above example).

If these criteria are not met, then level 3 or S₃ testing must be performed by testing 12 additional tablets. To pass S₃, the average of all 24 tablets must be equal to or greater than Q (80% LC; 160 mg in the above example). Two additional criteria must be met as well: 1) no more than 2 tablets are less than Q – 15% (65% LC; 130 mg in the above example), and 2) no tablet is less than Q – 25% dissolved (55% LC; 110 mg in the above example.)

#### 6.5.2 DELAYED-RELEASE DOSAGE FORMS

An aliquot of the acid medium from each vessel is analyzed at the end of the acid stage. For the acid stage, the acceptance criteria have three levels. Level 1 (A₁) testing is passed if no individual value exceeds 10% dissolved. If the A₁ criteria are not met, then the dissolution test is performed on 6 additional dosage forms for level 2 (A₂) testing. Level A₂ criteria are passed if the average of all 12 dosage forms in the acid stage is NMT 10% dissolved and if no individual dosage form is more than 25% dissolved. Level 3 testing is performed if the A₂ criteria are not met. The A₃ criteria are passed if the average of all 24 dosage forms in the acid stage is NMT 10% dissolved and if no individual tablet is more than 25% dissolved. For the special case in which the solubility of the drug in an acidic medium because of conversion to the free acid is too low to support an acceptance criterion of not more than 10% the drug product should be exposed to the acid stage for the defined duration and then exposed to the buffered medium. Alternate acceptance criteria for the acid stage based on drug solubility may be justified.

For delayed-release dosage forms, the total percentage dissolved is determined by adding the measured amounts in the acid and buffer phases for each individual dosage form. These calculated values are then compared to staged acceptance criteria.
(B₁, B₂, and B₃) that are based on a Q value. The B₁, B₂, and B₃ criteria are identical to those for the immediate release S₁, S₂, and S₃ criteria.

### 6.5.3 EXTENDED-RELEASE DOSAGE FORMS

In the following hypothetical example, which is used to describe the criteria for an extended-release dosage form, the time points are 1, 4, and 8 h. The acceptance range for each time point is as follows:

- Between 24% and 44% LC drug substance dissolved at 1 h
- Between 56% and 76% LC drug substance dissolved at 4 h
- NLT 85% LC drug substance dissolved at 8 h.

Acceptance ranges are often expressed in tabular form in the USP–NF (see Table 3):

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Amount Dissolved</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>24%–44%</td>
</tr>
<tr>
<td>4</td>
<td>56%–76%</td>
</tr>
<tr>
<td>8</td>
<td>NLT 85%</td>
</tr>
</tbody>
</table>

Six tablets are analyzed at Level 1 (L₁); acceptance criteria are met if no individual value lies outside each of the stated ranges, and no individual value is less than the percentage specified for the final time point. If the L₁ criteria are not met, then 6 additional tablets are analyzed at level 2 (L₂). The L₂ criteria are met if these three conditions are met:

1. The average value of the 12 tablets lies within each of the stated ranges and is NLT the stated range of the final time point.
2. None of the 12 tablets is >10% of the labeled content outside each of the stated ranges.
3. None of the 12 tablets is >10% of the labeled content below the stated amount at the final test time.

For the above example, the L₂ acceptance criteria for the 12 tablets (see Table 4) are as follows:

<table>
<thead>
<tr>
<th></th>
<th>1 h</th>
<th>4 h</th>
<th>8 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average</td>
<td>24%–44%</td>
<td>56%–76%</td>
<td>NLT 85%</td>
</tr>
<tr>
<td>Individual tablets</td>
<td>14%–54%</td>
<td>46%–86%</td>
<td>NLT 75%</td>
</tr>
</tbody>
</table>

If the L₂ criteria are not met, then 12 additional tablets are tested at level 3 (L₃). The L₃ criteria are met if these five conditions are met:

1. The average value of the 24 tablets lies within each of the stated ranges and is NLT the stated range of the final time point.
2. NMT 2 of the 24 tablets are >10% of labeled content outside each of the stated ranges.
3. NMT 2 of the 24 tablets are >10% of the labeled content below the stated amount at the final test time.
4. None of the 24 tablets is >20% of the labeled content outside each of the stated ranges.
5. None of the 24 tablets is >20% of the labeled content below the stated amount at the final test time.

The L₃ acceptance criteria for the 24 tablets in the above example are summarized in Table 5:

<table>
<thead>
<tr>
<th></th>
<th>1 h</th>
<th>4 h</th>
<th>8 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average</td>
<td>24%–44%</td>
<td>56%–76%</td>
<td>NLT 85%</td>
</tr>
<tr>
<td>Individual Tablets</td>
<td>NMT 2 tablets are outside the range of 14%–54%, and no individual tablet is outside the range of 4%–64%</td>
<td>NMT 2 tablets are outside the range of 46%–86%, and no individual tablet is outside the range of 36%–96%</td>
<td>NMT 2 tablets release &lt;75% and no individual tablet releases &lt;65%</td>
</tr>
</tbody>
</table>

### REFERENCES


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INTRODUCTION

This general information chapter provides a high-level description of principles for immunological test methods (ITMs) that can be used in specified monograph tests, along with information and approaches to analytical development and validation for ITMs. The scope of this chapter is to provide general information that is applicable to all ITMs. The chapter provides a foundation for specific chapters about different types of ITMs, e.g., Immunological Test Methods—Enzyme-Linked Immunosorbent Assay (ELISA) (1103), Immunological Test Methods—Immunoblot Analysis (1104) (proposed), and Immunological Test Methods—Surface Plasmon Resonance (1105). This suite of general information chapters is related to the bioassay general information chapters. Use of ITMs for process monitoring, diagnosis, and evaluation of clinical response, assessment of pharmacokinetics/pharmacodynamics/absorption, distribution, metabolism, and excretion (PK/ADME), and other product characterization (nonrelease testing) is outside the scope of this chapter.

The basis of all ITMs used to measure a quality attribute of a biologic drug substance or drug product is the highly specific noncovalent binding interaction between an antibody and antigen. The antigen typically is an analyte of interest (e.g., protein, carbohydrate, virus, or cell), and the binder is usually an antibody (e.g., monoclonal antibody or polyclonal antiserum). ITMs are applicable to molecules that are either directly antigenic (immunogens) or can be rendered indirectly antigenic (haptns). The measurand in ITM is directly related to a quality attribute of the product under test.

ITMs are valuable because they exhibit high sensitivity and specificity for an analyte in complex matrices. They typically are used for qualitative and quantitative assessment of both an antibody and antigen, but their application also extends to the measurement of hapten, complement, antigen–antibody complexes, and other protein–protein interactions. These properties of ITMs allow their use for assessing identity, potency (strength), purity, impurities, stability, and other quality attributes of biological drug substances and drug products.

ITMs are useful for many applications because they can measure molecules over a wide range of sizes and binding types. In general, antibodies are stable during various chemical modifications that do not have a significant adverse influence on interactions with an antigen. Antibody molecules tend to withstand moderate acidic and alkaline pH changes better than other
proteins do. Because of this characteristic, a variety of ITMs with high degrees of sensitivity and specificity are possible. The ability to accelerate contact between an antigen and antibody enables ITM formats that provide rapid or real-time results. Generally, ITMs have higher precision and shorter turn-around time than do traditional biologically-based (i.e., cell-based and animal) assays. Although in some cases these advantages can support the replacement of a biological assay with an immunoassay, such changes should be approached systematically and with caution. Often it is challenging to prove the equivalence, or comparability, of results from bioassays and immunoassays because the interaction between antigen and antibody may not reflect the functional attributes observed in bioassays.

One major limitation of ITMs compared to physicochemical methods (such as liquid or gas chromatography) is that the latter generally are more precise and can simultaneously identify a set of impurities or unexpected substance(s). Another major limitation is that generally ITMs operate at high molar dilutions at which they are sensitive to disturbances caused by environmental factors in the sample matrix (i.e., matrix effects). Matrix effects can depend on ITM format and are not fully understood. Their specificity, a hallmark of ITMs, is sometimes compromised by structural or sequence similarities between the analyte and a closely related molecular impurity (cross-reactivity).

Most ITMs reflect physical interaction (binding) between an antigen and antibody and not the analyte's functional properties. Therefore, analysts must pay attention in the selection and execution of ITM format. Cell-based ITMs that can provide functional information about the analyte are beyond the scope of this chapter.

**GENERAL CHARACTERISTICS OF ITMS**

ITMs are based on the principle of specific, noncovalent, and reversible interactions between an antigen and antibody. In general, the primary antigen–antibody reaction is brought about by complementarity, which creates macromolecular specificity. This noncovalent interaction determines the degree of intrinsic affinity. Intrinsic affinity contributes to functional and/or relative affinity that depends on factors like reaction phase and valency, which in turn determines the degree of reversibility of an interaction. A better understanding of factors that affect antigen–antibody interactions provides the rationale for the development of a suitable ITM format (e.g., solid or liquid phase, competitive or noncompetitive binding, etc.).

A defining characteristic of ITMs is that they employ an antigen (or hapten) and antibody. In addition, ITMs may contain companion molecules such as complement components. The components of ITMs are defined as follows:

- **Antigens**—Comprise a wide range of molecules that are capable of binding to the antibody in a specific interaction. Generally, part(s) of an antigen (the immunogenic epitope(s)) is/are capable of eliciting antibody response.
- **Haptens**—Small molecules that, by themselves, are not capable of eliciting an antibody response but are capable of eliciting an immune response when attached to a large carrier such as a protein. Antibodies produced to a hapten–carrier adduct also may bind to the small-molecule hapten in a specific interaction.
- **Complements**—Companion molecules that, under certain conditions, aid in the functionality of antigen–antibody complexes but are not required for antigen–antibody or hapten–antibody interaction.
- **Antibodies**—Proteins with regions that impart a high degree of specific binding to antigens (and haptens). The structural elements of an immunoglobulin G (IgG) antibody are shown in Figure 1.

In addition to these components, ITMs require some means to detect or monitor the binding reaction between the antigen and antibody.
Figure 1. The structure of IgG. The IgG molecule is characterized by a distinctive domain structure of heavy (H) and light (L) chains, both of which are divided into variable and constant regions (V and C, respectively). Light chains consist of $V_L$ and $C_L$ domains, and heavy chains consist of a variable domain ($V_H$) and three constant domains ($C_H1$, $C_H2$, and $C_H3$). All domains are stabilized by disulfide bonds, and $C_H2$ domains contain carbohydrates. The flexible hinge region between the $C_H1$ and $C_H2$ domains allows the independent behavior of two antigen-binding sites formed by variable domains.

**TYPES OF ITMS**

Measurement of antigen–antibody binding can be performed in a variety of assay types and formats: solid or liquid phase, manual or automated, labeled or nonlabeled, competitive or noncompetitive, qualitative or quantitative, homogeneous or heterogeneous, or combinations of some of these. The distinguishing characteristic of all these assays is the binding of an antibody or antigen to the analyte (which can be an antigen or antibody as well), followed by detection of the antigen–antibody complex. Although many different formats can be used for the binding reaction, along with different methods for detection, quantification of the analyte in the test article is always performed by comparison of the measurement to a reference standard. Thus a number of ITM technologies support investigations of product quality. Commonly used assay designs include enzyme-linked immunosorbent assay (ELISA), Western blotting, flow cytometry, competitive enzyme-linked immunosorbent assay, surface plasmon resonance (SPR), rate nephelometry, radioimmunoassay (RIA), radial immunodiffusion, precipitation, and agglutination. These methods are described below.

**Enzyme-Linked Immunosorbent Assay**

An ELISA is a quantitative, solid-phase immunological method for the measurement of an analyte following binding to an immunosorbent and its subsequent detection using enzymatic hydrolysis of a reporter substrate either directly (analyte has enzymatic properties) or indirectly (e.g., horseradish peroxidase– or alkaline phosphatase–linked antibody subsequently bound to the immunosorbed analyte). The analyte usually is quantitated by interpolation against a standard curve of a reference material. General information chapter *Immunological Test Methods—Enzyme-Linked Immunosorbent Assay (ELISA)* (1103) discusses ELISA in greater detail, including ELISA development for quantitative analysis.

**Western Blotting**

A Western blot is a semiquantitative or qualitative method for measurement of a protein analyte that has been resolved by polyacrylamide gel electrophoresis and subsequently transferred to a solid membrane (e.g., nitrocellulose, nylon, or polyvinylidene difluoride). Detection can be achieved directly by reacting with a labeled primary antibody (antibody specific to the analyte of interest) or indirectly by reacting labeled secondary antibody (antibody against the primary antibody) to the primary antibody bound to the membrane-immobilized antigen. The label can be a radioisotope or an enzyme that uses the substrate to produce color, fluorescence, or luminescence. This method is semiquantitative, especially when proteins are present in low concentration and in very complex mixtures. It is commonly used in early process development (e.g., antibody screening, protein expression, protein purification, etc.). Western blotting is a powerful method for analyzing and identifying proteins in...
complex mixtures, particularly after separation using 2-dimensional gel electrophoresis, which separates proteins based on size and charge (pI).

**Flow Cytometry**

Flow cytometry is a laser-based semiquantitative technology that permits measurement of fluorophore-conjugated probes as they interact with their respective ligands on cells or particles. More details for flow cytometry can be found in *Flow Cytometry* (1027).

**Surface Plasmon Resonance**

SPR is a quantitative method for measurement of an analyte in a sample where the antibody–antigen complex formation can be measured in real time at the interface of a liquid and solid (e.g., gold surfaces or particles). The measurement taken is the real-time change in refraction of a polarized light and occurs during the formation of the antibody–antigen complex, resulting in changes to the plasmon resonance minima (i.e., the sensorgram). The quantity of analyte is determined by comparison to the measurement of a reference standard curve determined in the same assay. More details for SPR can be found in general information chapter *Immunological Test Methods—Surface Plasmon Resonance* (1105).

**Rate Nephelometry**

Rate nephelometry is a quantitative method for measurement of an analyte in a sample in solution by measuring the light scatter introduced by small aggregates formed by the antigen–antibody complex. The quantity of analyte is determined by comparison to the measurement of a reference standard curve determined in the same assay.

**Radioimmunoassay**

RIA, a sensitive ITM first developed in the 1950s, is a quantitative method for measurement of an analyte in a sample. RIA usually uses a competitive antibody–antigen binding reaction, but it also can be used in sandwich immunoassay format, including immunoprecipitation. In competitive RIAs the analyte competes for binding with a radiolabeled (e.g., using $^{125}\text{I}$ or $^{3}\text{H}$) reference antigen that is identical to the analyte; therefore, the analyte and the antigen both compete for binding to a fixed and limiting dilution of a specific (often polyclonal) antibody. The radiolabeled antigen is present in excess. The same unlabeled antigen in the test sample competes in binding to the same site on the antibody, which is present in a fixed quantity. Binding of the unlabeled antigen to the antibody leads to the displacement of the labeled antigen, resulting in a decrease in the radioactivity of the antigen–antibody complex fraction. To separate the antigen–antibody complex from the excess unbound antigen, the complex generally is either precipitated with a secondary antibody (or protein G) immobilized on a solid matrix (e.g., glass or resin beads) or with an already immobilized primary antibody. The quantity of analyte usually is determined by interpolation against a standard curve of a reference material, where a fixed amount of antibody and radiolabeled antigen is mixed with an increasing amount of unlabeled antigen. Hence, even a small quantity of unlabeled antigen will result in a relative quantitative decrease in total bound radioactivity.

**Single Radial Immunodiffusion**

Single radial immunodiffusion (SRID or SRD) is a quantitative method for measurement of an analyte in a sample by measuring the diameter of the ring of precipitin formed by the antigen–antibody complex. Antigen is applied to a well in a gel infused with a constant level of antibody. Solutions with higher concentrations of antigen diffuse farther before being saturated with antibody and then precipitated. The quantity of analyte is determined by comparison to a reference standard curve measured by the same assay.

**Precipitation**

The underlying principle for this method is that the interaction of a multivalent antibody and antigen leads to the formation of a complex. In some cases a visible precipitate is formed. Other immunoprecipitation techniques involve the use of Protein A or Protein G beads to capture the antigen–antibody complex and facilitate the separation of the antigen–antibody complexes from the other antigens in the solution. Precipitation is not commonly used for quantitative analytical purposes because of the time required (days to complete), lack of sensitivity, and requirement for large quantities of antigen and antibodies.

**Agglutination**

Agglutination and inhibition of agglutination, respectively, provide qualitative and quantitative measures of certain antigens and antibodies. Inhibition of agglutination is a modification of the agglutination reaction that provides higher sensitivity to detect small quantities of proteins, chemicals, viruses, and other analytes. The principle of agglutination is similar to that for precipitation except that the interaction takes place between an antibody and a particulate antigen and leads to a visible clump or agglutination. The most common example of this application is for blood typing (i.e., A, B, or O antigen).
**CHOICE OF ITM**

When choosing an ITM, analysts should consider sensitivity and specificity as well as the complexity of the sample. *Table 1* provides an assay developer with a comparative view of the advantages and disadvantages of a variety of ITM formats. The intended application of the ITM should govern the choice of the most suitable format.

*Table 1. ITMs Used in Biopharmaceutical Laboratories*

<table>
<thead>
<tr>
<th>Method</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Typical Industry Uses</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELISA</td>
<td>• High sensitivity</td>
<td>• Multistage process highly dependent on proper execution of each stage</td>
<td>• Potency assessment</td>
</tr>
<tr>
<td></td>
<td>• Often wide dynamic range</td>
<td>• Wash steps add time and often biohazardous waste</td>
<td>• Specific protein concentration analysis in complex samples</td>
</tr>
<tr>
<td></td>
<td>• High throughput</td>
<td>• Reagent labeling required</td>
<td>• Protein identification</td>
</tr>
<tr>
<td></td>
<td>• Low cost</td>
<td></td>
<td>• Purity assessment</td>
</tr>
<tr>
<td>Western blot</td>
<td>• Gives information about antigen size and/or charge</td>
<td>• Typically works only with linear epitopes</td>
<td>• Immunogenicity assessment</td>
</tr>
<tr>
<td></td>
<td>• Allows separation of various antigens (or degradation/aggregation products) bearing same epitope</td>
<td>• Labor intensive</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Can tolerate complex mixtures</td>
<td>• Low throughput, output</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Subject to interpretation</td>
<td></td>
</tr>
<tr>
<td>Flow cytometry</td>
<td>• High throughput</td>
<td>• Immobilization can alter binding</td>
<td>• Protein purity assessment</td>
</tr>
<tr>
<td></td>
<td>• Highly automated</td>
<td>• Limited to proteins</td>
<td>• Protein stability assessment</td>
</tr>
<tr>
<td>SPR</td>
<td>• Direct detection of binding</td>
<td>• Regeneration can alter binding</td>
<td>• Protein identity test</td>
</tr>
<tr>
<td></td>
<td>• Can measure affinity precisely, including on and off rates</td>
<td>• Low throughput, output</td>
<td></td>
</tr>
<tr>
<td>Rate nephelometry</td>
<td>• Easily automated</td>
<td>• Small detection range</td>
<td>• Assay for individual vaccine components for check of stability and purity</td>
</tr>
<tr>
<td></td>
<td>• Rapid</td>
<td>• High background for turbid samples</td>
<td></td>
</tr>
<tr>
<td>RIA</td>
<td>• Binding occurs in native conformation</td>
<td>• Requires radioactive labeling for detection</td>
<td>• Protein identification (e.g., hormones)</td>
</tr>
<tr>
<td></td>
<td>• Low-concentration samples can be analyzed</td>
<td>• Shorter half-life of some radioisotopes requires periodic preparation of the tracer</td>
<td>• Specific protein concentration analysis in complex samples</td>
</tr>
<tr>
<td></td>
<td>• High sensitivity antibody used at limiting dilution that conserves reagent</td>
<td>• Hazardous waste</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Can be plate-based for higher throughput (e.g., scintillation proximity assays)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SRD</td>
<td>• Precise</td>
<td>• Semiquantitative</td>
<td>• Vaccine release test</td>
</tr>
<tr>
<td></td>
<td>• Simple setup</td>
<td>• Low precision</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Low sensitivity</td>
<td></td>
</tr>
<tr>
<td>Precipitation</td>
<td>• Low equipment cost</td>
<td>• Subject to interpretation</td>
<td>• Vaccine identification</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Slow</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Poor sensitivity (μg range)</td>
<td></td>
</tr>
<tr>
<td>Agglutination</td>
<td>• Rapid</td>
<td>• Subject to interpretation</td>
<td>• Vaccine identification</td>
</tr>
<tr>
<td></td>
<td>• Low equipment cost</td>
<td>• Slow</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Low specificity because of interfering substances</td>
<td></td>
</tr>
</tbody>
</table>

**KEY CONSIDERATIONS IN ITM DEVELOPMENT**

The goal during method development is to produce an accurate assay that is practically feasible and possesses an acceptable degree of intra- and inter-assay precision. To minimize the overall imprecision, the sources of variability should be identified and minimized.

**Reagent Selection**

Imunoassays are subject to several sources of interference such as cross-reactivity, endogenous interfering substances, buffer matrices, sample components, exposed versus masked epitopes, conformation changes in the antigen of interest, and other factors. Hence, during method development, analysts must identify possible sources of interference both to develop a robust method and to aid future troubleshooting. Cross-reactivity is a major obstacle during immunoassay development. This arises when the specificity of an antigen–antibody reaction is compromised by the cross-reactivity binding of structurally similar molecules with the reaction binder. Some common
examples are protein isoforms, degraded analyte entities, molecules of the same class, precursor proteins, metabolites, etc. Cross-reactivity can be minimized by rigorous reagent characterization and selection. Reagents used in ITM applications generally fall into one of two categories: critical reagents and noncritical reagents. Critical reagents are specific and unique to the particular ITM or reagents that are intolerant of very small changes in composition or stability. Examples of critical reagents generally include assay-specific antibodies and reference or method calibration standards. Equivalence in the assay format must be established before replacement with a new lot. Noncritical reagents are those that can vary to some degree in composition without adversely affecting ITM performance. Reagents are often assumed to be noncritical (e.g., buffers, water quality, blocking buffer, or substrate) but later may be identified as critical components if assay ruggedness fails and troubleshooting of ITM reagents begins. ITM-specific reagents, including vendor and catalog number, should be defined in test procedure documents. Antibody selection is critical for development of a successful immunoassay because it defines the assay’s specificity and sensitivity. Furthermore, during antibody generation, analysts should ensure that the immunization protocols support the end use of the antibodies. For some applications a more specific antibody can be generated by the selection of a small and specific immunogen and affinity purification of the antibody, resulting in highly defined epitope coverage. In other applications it may be critical to ensure broad coverage of the different available epitopes on the molecules of interest, and a polyclonal antibody (pAb) pool may be the best choice. Currently, monoclonal antibodies (mAb) are preferred for some applications for the detection of single analytes because of their high specificity, lot-to-lot consistency, and indefinite supply. Compared to polyclonal antibodies, mAb have a higher initial cost to produce, but for these applications, the advantages generally outweigh the initial cost. Other applications may require more comprehensive epitope selection to ensure that subtle changes in the molecule(s) do not prevent recognition of the entire antigen, and thus a pool of monoclonal antibodies, or a pAb pool, would be the preferred choice. The latter are widely used for detection in a complex mixture of analytes (e.g., host-cell proteins). Similarly, immunoassays may use two distinct epitopes on an antigen—one for capture and the other for detection—which greatly reduces cross-reactivity. Another approach to minimize cross-reactivity is to purify the antigen before immunoanalysis. Variations in incubation temperature and time can affect the reaction kinetics of antibody interactions with similar yet different antigens. Thus this property should be optimized to increase the specificity of antigen–antibody interactions.

**Development of Immunoassays**

Development is an important stage in the establishment of a suitable ITM. During development of an ITM, analysts explore various settings of assay parameters and interactions between parameters to identify conditions under which the assay will consistently produce reliable results using minimal reagents, effort, and time. In Quality by Design terminology, the “possible operating space” is the collection of settings of assay parameters explored, and the “design space” refers to the conditions under which the assay performs well. The necessary performance properties of the ITM (precision, accuracy, specificity, etc.) required depend on the intended use(s). During ITM development, analysts should consider the following:

- Antigen–antibody ratio;
- In sandwich immunoassays, the ratio of capture antibody to detector antibody;
- Antigen–antibody reaction kinetics in the sample matrix (antigen–antibody binding generally is not linear);
- Selection of the standard (full-length antigen for the standard or just a small portion of the antigen containing the antibody-binding epitope, among other considerations); and
- Matrix effects.

The use of design of experiments (DOE) is strongly recommended, and different DOE methods may be appropriate in each stage of development. Early in development, screening designs are particularly useful (generally two-level geometric fractional factorial designs). After screening (with a modest number of factors to study), full factorials or response surface designs are often appropriate. As development activities shift to qualification (ideally, if not typically, as the focus shifts to robustness), robust response surface designs often are a good choice. During qualification or validation, analysts may find it practical to simultaneously study robustness to assay operating conditions (using a small geometric fractional factorial) and validation parameters such as precision (via nested or crossed designs for random factors associated with repeatability, intermediate precision, and reproducibility).

Experiments that assess dilutional linearity and components of specificity, including matrix effects, usually involve construction of spiked samples. Although spiking often is performed in a dilution matrix, spiking a collection of actual samples or mixing actual samples is an important component of demonstrating robustness of dilutional linearity and components of specificity to the sample and matrix components.

**Reagent Considerations**

A procedure for qualifying reagent sources and vendors (including audits), ordering, receiving, and disposing of commercial reagents and consumables should be outlined in a standard operating procedure (SOP). The preparation of internal reagents must be documented in a manner that allows reconstruction. Commercial and internally prepared reagents must be labeled with identity, concentration, lot number, expiration, and storage conditions. The stability and assignment of expiration dates for internally prepared reagents often are based on available literature and scientific experience, but analysts may need to confirm these empirically. An SOP for extending expiration dating of critical reagents is recommended. In addition, analysts should implement a mechanism for reagent tracking and linking lot numbers to analytical run numbers. Unacceptable reagent performance is detected by tracking QC samples. Shifts in QC samples should prompt a review of analytical runs and changes in reagent lot numbers or review of possible deterioration of critical reagents. To avoid such shifts, analysts can cross-validate critical reagent lot changes.

The impact of collection and storage containers on analytical performance often is overlooked. When defining the stability and expiration of in-house reagents, analysts should record information about the storage container vendor, catalog, and lot number. The importance of a suitable reference standard and its characterization cannot be overemphasized for ITMs for
biological products. Because of their inherent complexity, reference and calibration standards of macromolecular biologics often are less well characterized than are conventional small-molecule drug reference standards. If the calibration standard represents a mixture of different antigens (e.g., host-cell proteins), it should be shown to be representative of the antigen profile in the samples being tested. Consistency in ITM results depends on the availability of a suitable representative reference standard material.

**VALIDATION**

Analytical validation involves the systematic execution of a defined protocol and prespecified analysis that includes prespecified acceptance criteria. A validation demonstrates that an analytical method is suitable for one or more intended uses [see Validation of Compendial Procedures (1225), Biological Assay Validation (1033), and ICH Q2(R1)]. Qualification may involve similar or identical experiments and procedures as validation, but qualification does not require prespecified protocols, analyses, or acceptance criteria. In certain situations (e.g., use of a commercial kit), assay development may not be required before qualification. General information chapter (1225) discusses which assay performance characteristics must be examined during validation for four primary categories of intended uses. For example, analytical procedures that quantitate major bulk drug substances or active ingredients may not require validation of the detection and quantitation limits but do require validation of accuracy, precision, specificity, linearity, and range.

**System Suitability or Assay Acceptance Criteria**

The purpose of system suitability or assay acceptance criteria is to ensure that the complete system—including the instrumentation, software, reagents, and analyst—is qualified to perform the intended action for the intended purpose. All processes should be controlled by well-defined SOPs that ensure consistency, reduce errors, and promote reproducibility of laboratory processes. Training files for all personnel should be contemporaneous and should include some demonstration that analysts are qualified to perform the method and the specific ITM.

Instrument and software qualification begins with a definition of the design qualifications, including a risk assessment and gap analysis that identify potential threats to the collection, integrity, and permanent capture of ITM data. Qualification also includes installation qualifications (IQ) and operational qualifications (OQ). Purchased commercial instrument validation packages may require modification to meet the intended use at each facility. Instrumentation and software should be continuously monitored for acceptable functionality by performance qualification (PQ) and software validation test script reviews. Routine instrument maintenance is performed according to the manufacturer’s recommendations, and additional maintenance may be required based on specific needs in the working environment. A complete history of routine and nonroutine instrument maintenance should be archived for each instrument. Software updates should be handled with change control and typically require additional validation. Adherence to 21 CFR 11 should be maintained.

To ensure robustness, establish a defined process for implementing new ITMs in the laboratory. Control documents should be in place, including method validation plans containing a priori method acceptance criteria and validation reports for the establishment of a new ITM. Well-written analytical test method documents are needed to ensure reconstruction of analytical results and to minimize laboratory errors.

Analytical test methods should include acceptance criteria for critical aspects of the assay, including the performance of the calibration curve, quality controls, agreement between sample replicates, procedures for repeat sample analysis, and identification and treatment of outliers, when applicable. Furthermore, an SOP should be implemented for unexpected event investigation and resolution.

**DATA REPORTING**

**Units of Measurement**

Quantitative ITMs generate test sample data with an estimated concentration based on a calibration curve fit to reference (or standard) samples using an appropriate mathematical model. When determining the amount of analyte in a manufacturing process, analysts often express the unit of measure in terms of mass of analyte per volume of solution (concentration) or mass of analyte per mass of product (e.g., parts per million). Depending on the nature of the measured analyte, the degree of measurement standardization, the geographic region, and the history of the method, analysts may express concentration in terms of weight per volume, mole per volume, or weight of analyte per weight of product. In some circumstances, concentration may be converted to an activity unit of measure in which the analyte mass is assumed to be 100% active. In certain circumstances, qualitative analysis using a predetermined cut-off value may be an acceptable alternative to quantitative methods.

**Immunoassay Data Analysis**

ITMs employ calibration curves prepared with reference standards of known (nominal) concentrations and are included in every bioanalytical method. This helps control variation associated with repeatability, intermediate precision, and reproducibility and permits the estimation of results for unknown test samples. Common simple statistical analyses assume that the (possibly transformed) data are normally distributed, have constant variance, are independent, and that an appropriate model has been used. For many assays, one or more of these assumptions may be inappropriate. Analysts should assess these assumptions using a substantial body of data (typically tens of assays). When these assumptions are not reasonable, the analysis becomes more complex.
Calibration curves generally are characterized by a nonlinear relationship between the mean response and the analyte concentration and typically are plotted in a log-linear manner with the (possibly transformed and/or weighted) response variable (ordinate) plotted against the nominal calibrator concentration (abscissa) in log scale. The resulting curve that encompasses the assay’s validated range is inherently nonlinear and often has a sigmoid shape with horizontal asymptotes at very low and high concentrations of analyte. Competitive ITMs have a negative slope, and noncompetitive ITMs are characterized by a positive slope. The analyte concentration in a test sample is estimated by inverse regression against the calibration curve. The final result often is obtained after multiplication of the estimated concentration in the assay by a dilution factor that is required to yield a response within the ITM’s quantification range.

Under the guidance of a qualified biostatistician, analysts can implement outlier tests in controlled documents that permit the exclusion of spurious sample results. A well-defined procedure should be in place regarding how to identify, repeat, and report outliers. Outlier tests and interpretation of results are described in Analytical Data—Interpretation and Treatment (1010). Test results that fall outside of their predefined specifications or acceptance criteria should be evaluated by an out-of-specification investigation to identify a root cause.

### Trending

A quality system includes monitoring of ITM performance by collection and review of ITM performance characteristics. Trending may detect shifts in assay performance that may be related to events such as assay reagent lot changes, addition of new analyts, shifts in environmental conditions, and others. SOPs, study protocols, analytical test methods, and decision flow charts are recommended to strictly define the handling, use, editing, rejection, acceptability, and interpretation of calibration data and test sample results for ITMs. It is not uncommon to have several raw data reviews, including peer, QC, and quality assurance review. Analysts must be able to distinguish such analytical issues from true changes in the measured analyte caused by changes or errors in the manufacturing process that have affected the product. Two of the most important outcomes of trend are detecting potential problems before they occur and identifying areas for corrective and/or preventive action. General information chapters Analytical Data—Interpretation and Treatment (1010) and Biological Assay Validation (1033), as well as the statistical literature, contain guidance for various trending methods. Several ITM performance characteristics could be considered for monitoring. The most common trending value is evaluation of QC samples. Ideally, one or more QC sample is available for long-term trending in sufficient quantity and with demonstrated stability so that quality aspects can be assayed in every run and across multiple manufacturing lots. As the long-term QC sample is depleted or expires, crossover comparison and establishment of a new long-term QC sample should be completed. Systematic review of QC data across assays assists in troubleshooting failed ITM runs, providing confidence in the evaluation of spurious results, and controlling the introduction of replenished assay components that may not perform exactly like previous reagents.

Other ITM performance characteristics that may be monitored include calibration curve response variables, curve fit parameters, assay background, and comparison of in-study QC data with validation data.

### Tracking

Regulatory agencies have strict requirements about maintaining the identity and integrity of both samples and data. A quality process driven by SOPs must be implemented to ensure the correct identity and integrity of test and reserve samples. Ideally, a bar code system should be used to track the collection, identity, location, chain of custody, number of sample freeze/thaw cycles, storage temperature, and length of time that a sample is stored. This information should be captured and should be auditable from the time of collection to disposal (or sample depletion). The ability to track the sample history permits reconstruction of the events leading to generation of a data result. This information is used by regulatory agencies to ensure that the proper procedures were followed and by internal auditors to ensure that pre-analytical sample handling did not compromise study data. In addition, sample tracking allows a mechanism for ensuring that the analyte measurement occurred within the demonstrated window of stability for that analyte. The final result generated from a bioanalytical laboratory is a number that represents an analyte measurement in a test sample. The steps necessary to generate that data and preserve it in a report are numerous and are susceptible to error. Therefore, quality systems must be in place to minimize data errors. Errors may be introduced by test sample misplacement or identification, incorrect data reduction, miscalculations, transcription errors, omissions, and other factors. Ideally, validated software and laboratory information management systems are used when possible to generate, transfer, and archive data. Typically, redundancy checks are built into automated processes by visual data review of at least 10% of the data-transfer processes. In the absence of validated electronic transfer, all data should be reviewed by at least one reviewer. As with sample tracking, data generation, manipulation, and storage should be reconstructable. In addition, sample tracking allows a mechanism for ensuring that the analyte measurement occurred is stable. Plans should be in place to update archived data so that, as technology changes, archived data can still be retrieved. Regulatory agencies require that raw data be available for various lengths of time after the completion of a study or regulatory filing. Finally, data must be secure from corruption, alteration, or access by unauthorized personnel.
IMMUNOLOGICAL TEST METHODS—ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

INTRODUCTION

Immunological test methods (ITMs) utilize bindings between an antigen (Ag) and antibody (Ab). (See Appendix 1: Abbreviations for a complete list of acronyms used in this chapter.) Enzyme-linked immunosorbent assay (ELISA) is one of the most widely used ITMs for characterization, release, and stability testing of biotechnology products to help ensure the quality of biological drug substances and drug products. The term “ELISA” is used here in a broader sense and includes enzyme immunoassays (EIAs), as well as alternative detection methods, e.g., chemiluminescence and fluorescence.

This chapter provides analysts with general information about principles, procedures, experimental configurations, assay development, and validation for solid-phase ITMs like ELISA and can be used for the other immunoassay variations mentioned above. The chapter also covers reference standards and controls used for immunoassays. The information can be adapted to the specific procedures of a monograph. This chapter does not cover immunoassays for the measurement of immune responses to product in animals or humans (e.g., serological or cellular assays), non-immunoassays (e.g., receptor-ligand interactions), or other related approaches.

The chapter is part of a group of general information chapters for ITMs (Immunological Test Methods—General Considerations (1102), Immunological Test Methods—Immunoblot Analysis (1104), and Immunological Test Methods—Surface Plasmon Resonance (1105)), and also is related to the general information chapters for bioassays (Design and Development of Biological Assays (1032), Biological Assay Validation (1033), and Analysis of Biological Assays (1034)).

Definition

ELISA can be defined as a qualitative or quantitative solid-phase immunological method to measure an analyte following its binding to an immunosorbent surface and its subsequent detection by the use of enzymatic hydrolysis of a reporter substrate, either directly (as with an analyte that has enzymatic properties or is directly labeled with an enzyme) or indirectly (by means of an enzyme-linked antibody that binds to the immunosorbed analyte). Qualitative results provide a simple positive or negative result for a sample. Converting quantitative to qualitative results based on a cutoff value that separates positive and negative results is common practice. Because the performance properties of the assay depend heavily on the cutoff value, the process used to determine the cutoff should be evidence-based and well documented. Quantitative assays determine the quantity of the analyte based on the interpolation of a standard calibration curve with known analyte concentration, run simultaneously in the same assay. This standard should be an appropriate, preferably homologous, reference or calibration material that is representative of the analyte(s) of interest. The power of immunoassays has been demonstrated by the variety of procedures that have evolved, including alternative solid surfaces such as beads of different sorts, various plastics in plates of different configurations, and alternative detection methods, e.g., chemiluminescence and fluorescence. ELISA assays are widely used in the biopharmaceutical industry for various applications such as identity, purity, potency, detection or quantitation of antibody or antigen, and other purposes.

Basic Principles

The essential steps of an ELISA can be broken down as follows (see Figure 1):
1. Binding of the capture reagent (generally an antibody or antigen), which functions as an immunosorbent for capture of the analyte, to a solid surface;
2. Removal of excess, unbound capture reagent followed by blocking of unoccupied binding sites with a blocking protein such as albumin, gelatin, casein, or other suitable material;
3a. Incubation of the analyte (in the test sample or reference standard) with the capture reagent to bind the analyte onto the solid surface, followed by the washing away of unbound material in the test sample and detection of the analyte.
3b. Incubation of the analyte (in the test sample or reference standard) with the capture reagent to bind the analyte onto the solid surface, followed by the washing away of unbound material in the test sample and subsequent detection of the analyte.
4. Quantification of the analyte by addition of a substrate suitable for the detector used [e.g., 3,3',5,5'-tetramethylbenzidine (TMB)], followed by comparison of the test sample to the reference standard.

ASSAY DESIGN

Four general categories of ELISA are described in Table 1 and in the sections that follow. The assay designs are flexible and, depending on specific needs, can be modified from these procedures. The choice of format depends primarily on the amounts and purity of reagents and equipment available. On some occasions the analyte being characterized actually is an antibody, as in the case of a monoclonal antibody that is being developed as a drug. In this case, anti-idiotypic or other antibodies specific for the antibody are used to develop the assays.

Change to read:

1 Capture reagent binding, blocking, analyte binding, detector antibody binding, and analysis are the five basic steps in an ELISA. Capture reagent binding, blocking, and analyte binding steps are each followed by a washing step to remove unbound reagents before the addition of the next reagent. Before analysis an appropriate substrate is added, followed by measurement of the substrate by appropriate equipment for detection. Quantitation of unknowns takes place by comparison with a standard curve.
Table 1. Representative ELISA Types

<table>
<thead>
<tr>
<th>ELISA Type</th>
<th>Required Reagents</th>
<th>Attributes</th>
<th>Disadvantages</th>
</tr>
</thead>
</table>
| Direct detection | • Capture analyte<sup>a</sup>  
• Labeled primary antibody specific for antigen | • Rapid because only one antibody is used  
• Uses less reagent  
• Analyte is immobilized | • May modify the conformation of the analyte  
• Sensitive to matrix and adjuvant components  
• Not commonly used  
• Poor sensitivity |
| Indirect detection | • Capture analyte<sup>a</sup>  
• Primary antibody specific for antigen  
• Labeled secondary detector antibody that binds to the primary antibody | • Versatile because a variety of primary antibodies can be used with the same secondary detector  
• Improved sensitivity because of signal amplification  
• Analyte is immobilized | • Longer because of more incubation and washing steps |
| Competitive | • Analyte<sup>a</sup> can be used as a capture reagent or can be labeled with a detection label  
• Antibody specific for analyte can be used for capture or labeled for detection  
• Labeled secondary antibodies to bind to primary antibody if an indirect format is used | • Good for assessing antigenic cross-reactivity  
• Appropriate for smaller proteins with single epitopes  
• Requires only a single antibody  
• Analyte in solution competes for binding to primary antibody | • Format difficult to troubleshoot  
• Limited dynamic range |
| Sandwich | • Primary capture antibody specific for analyte  
• Sample solution containing analyte<sup>a</sup>  
• A different primary enzyme-antibody conjugate specific for analyte | • Improved sensitivity  
• Good for quantitative assays for larger multi-epitope molecules  
• Analyte measured in solution | • Requires relatively large amounts of pure or semipure specific antibody  
• Not suited for smaller proteins that may have only a single epitope or a few closely spaced epitopes |

<sup>a</sup> This reagent can be either purified or partially purified. The terms “analyte” and “antigen” are used interchangeably when describing ELISAs.

**Direct ELISA**

**DIRECTLY LABELED ANTIBODY**

In this assay an antigen is coated onto a solid surface and the remaining unbound reactive sites are blocked [Figure 2 (A)]. Then a solution containing a specific antibody labeled with a detector is added. After incubation, the unbound antibody is washed away, followed by the addition of an appropriate substrate for the detector used.
Figure 2. Schematic representations of direct, indirect, competitive, sandwich, and bridging ELISAs.2 [Ab = antibody; Anti-Ig = anti-immunoglobulin; Ag = antigen (or analyte); Conc = concentration; Preincub = preincubation]

DIRECTLY LABELED ANTIGEN

This assay is similar to that using a directly labeled antibody, except that the antibody is coated onto the solid surface and a labeled antigen is used as the detector.

Indirect ELISA

In this assay an antigen is coated onto a solid surface and then, after blocking, a solution containing a specific antibody is added [Figure 2 (B)]. After incubation, the unbound antibody is washed away, followed by the addition of an anti-immunoglobulin (anti-Ig) detector antibody. Anti-Ig detectors are available commercially for specific Ig classes and subclasses from a variety of species, which makes this assay format useful for isotyping of antibodies. In addition, the use of a labeled anti-Ig detector amplifies the signal compared to a Direct ELISA, thereby increasing assay sensitivity.

Competitive ELISA

DIRECT ANTIBODY COMPETITIVE ELISA

This assay is used to detect or quantitate soluble antigens [Figure 2 (C)]. It requires an antigen-specific antibody that has been conjugated to an appropriate detector, e.g., horseradish peroxidase (HRP), alkaline phosphatase (AP), ruthenium, or fluorescein. It also requires a purified or partially purified antigen for coating. The antigen is coated onto a solid surface, followed by a blocking step. The antibody–conjugate is incubated with the test solution containing soluble antigen. The mixture is then added to the immobilized antigen, incubated, and unbound antigen–antibody complex is washed away. Substrate is added, and the inhibition of the reaction (e.g., colorimetric, electrochemiluminescence, fluorescence, or chemiluminescence) is measured relative to the reaction when no competitor antigen is added. The amount of inhibition is inversely proportional to the amount of antigen in the test sample. Competitive assays can also measure small molecules by coating an antibody to the plate that is specific to the small molecule. The small molecule is often biotinylated with a long linker that does not interfere with binding between the capture antibody on the plate and the small molecule. Antigen (the small molecule) in the sample then competes with the labeled small molecule for binding to the capture antibody. After washing, a detection reagent (e.g., streptavidin labeled with HRP) is added to detect the binding complex.

2 The type of ELISA format depends on the availability of reagents, the intended purpose of the assay, and the physicochemical characteristics of the analyte of interest. For a Bridging ELISA, the capture and detector antibodies recognize the same epitope, and therefore the target antigen must have at least two epitopes available for binding.
DIRECT ANTIGEN COMPETITIVE ELISA

This assay is similar to the Direct Antibody Competitive ELISA, except that it is used to detect soluble antibodies. The antigen is conjugated to the detector and the antibody is coated onto the solid surface.

INDIRECT ANTIBODY COMPETITIVE ELISA

This assay is similar to the Direct Antibody Competitive ELISA, except that instead of directly labeling the antibody, the test uses a labeled anti-Ig reagent for detection.

INDIRECT ANTIGEN COMPETITIVE ELISA

This assay is similar to the Direct Antigen Competitive ELISA, except that instead of directly labeling the antigen, the test uses a labeled secondary antibody for detection.

Sandwich ELISA

DIRECT SANDWICH ELISA

In this assay an antibody is immobilized onto a solid surface and blocked, and then a solution containing a specific antigen is added [Figure 2 (D)]. After an incubation step, the unbound material is washed away, and a labeled detector antibody is added. This assay format requires two antibodies, each of which binds to different epitopes on the surface of the large and complex molecule. The two antibodies are specific for the antigen, and the antigen should be sufficiently large and complex to accommodate the binding of two antibodies.

INDIRECT SANDWICH ELISA

Alternatively, instead of directly labeling the detector antibody, an anti-Ig antibody detector can be used. Indirect sandwich immunoassay formats can be considered only if each binding reagent is from a unique species (e.g., a sandwich assay using two mouse monoclonal antibodies for capture and detection could not be detected indirectly because the resulting signal may become independent of the antigen concentration).

BRIDGING ELISA

This subset of Sandwich ELISA assays often uses a single antibody for both capture and detection [Figure 2 (E)]. If a monoclonal antibody is used, it requires that the target antigen have at least two identical epitopes that are adequately spaced to prevent steric hindrance so that one epitope binds to the capture antibody and the other epitope binds to the detector antibody. Alternatively, a polyclonal antibody can be used but still requires that the target antigen be large enough to accommodate the binding of two antibody molecules. With respect to specificity and sensitivity, bridging assays usually are suitable for most large molecules.

CHOICE OF ASSAY

Deciding which ELISA procedure or format to use often depends on individual choice and availability of reagents, instruments, and other equipment. For example, sometimes a laboratory repeatedly engineers a particular epitope into multiple fusion proteins. In this case, the laboratory can use certain common qualified reagents (e.g., an antibody to a glutathione S-transferase region in multiple fusion proteins), facilitating rapid sandwich immunoassay development. Small antigens with a limited number of epitopes available for antibody binding restrict ELISA format choices. If there is only one binding epitope, then ELISA methods that use the sandwich/two-site binding or other bridging formats cannot be used because they require at least two available epitopes for antibody binding. In addition, small molecules are not usually used as a capture reagent on a plate because the process may interfere with binding to the detection reagent. Examples of such small molecules are some peptides, oligosaccharides, nucleotides, and antibacterials. Analysts usually adopt a competitive assay format for such small analytes.

Different assays and formats may demonstrate different properties and characteristics, e.g., specificity, precision, accuracy, sensitivity, dynamic range, dose-response ratio, sample throughput, sensitivity to interference, and simplicity or efficiency for automation. Ease of validation also may vary between different assay protocols and formats. Assay designs with replicates in adjacent wells could be biased if there are location effects; hence, in this case, replicates should not be in adjacent wells. Assay designs that are convenient to perform on 96-well plates, using relatively few single-channel pipet actions and more multi-channel pipet actions, are usually easier to adapt to automation. Assays with steep dose-response curves are generally better able to deliver high precision estimates; however, some assays with steep dose-response curves are imprecise in the EC_{50} and require a wider dose range.
PROCEDURES

Solid Phase

Solid phases are available in a variety of forms (e.g., membrane, plate, or bead) and chemistries [e.g., nylon, nitrocellulose, polyvinylidene fluoride (PVDF), polyvinyl, polystyrene, or a chemically derivatized surface]. The selection of the solid phase determines the most likely binding mechanism, i.e., hydrophobic, hydrophilic, or covalent interactions. In general, compared to plates, beads offer higher capacity and are more commonly used in clinical assays whereas plates are more commonly used to test biotechnology products. Additional information on plates is provided below.

COATING THE SOLID PHASE—IMMobilIZATION OF CAPTURE REAGENT

Capture reagents are coated onto a solid phase by adding a solution containing the capture reagent to the surface. The most commonly used solid-phase materials for capture reagent immobilization are plastic 96-well microtiter plates. Those with flat-bottom wells are recommended for spectrophotometric readings, and round-bottom well plates are useful for visual assessment of a dye’s color development. The degree of coating is influenced by the concentration of capture reagent, temperature during coating, duration of capture reagent adsorption, the surface properties of the solid-phase material, and the nature of the buffer of the capture reagent solution. Although the optimum coating concentration must be determined for each capture reagent, concentrations of 1–10 µg/mL are most commonly used. The volume of capture reagent added to each well usually corresponds to the sample volume that will be analyzed, i.e., 50–100 µL. Coating duration, temperature, and buffers are discussed separately below. During the coating procedure analysts should avoid introducing bubbles. Proteins that bind to plastic can be denatured, which alters antigenicity. In such cases, a capture antibody or an intermediary protein such as Protein A or Protein G can be used. In addition, streptavidin can be used if the reagent is biotinylated. The pH of the coating buffer should be optimized based on the isoelectric point of the capture reagent and the surface properties of the assay plate chosen.

MICROTITRER PLATES

The composition and commercial source of the microtiter plate can influence binding of the capture reagent during coating. Several microtiter plates from different suppliers should be compared using a single coating procedure to select those that provide high specificity for the capture reagent of interest and low nonspecific background. Comparisons of different grades of plates from a single supplier also may be needed. Clear plates typically are used for colorimetric ELISA, and opaque plates often are used for chemiluminescent and fluorometric ELISA. Acidic capture reagents may require a lower pH solution to neutralize repulsive forces between the protein and solid phase. Peptides often require optimization of buffer pH based on their charge for optimal coating conditions during assay development. Polysaccharides, lipopolysaccharides, or glycoproteins may be difficult to coat directly to the plate and may require a capture antibody or a buffer that contains lysine or glutaraldehyde. Coating with an antibody can be enhanced by precoating the microtiter plate with Protein A or Protein G or a combination of the two, which allows binding to the Fc region so that the Fab portion can bind to the analyte of interest. However, care must be taken to ensure that subsequent secondary antibodies do not react with the Protein A- or Protein G-coated wells. In this case, for example, chicken IgY or another appropriate antibody class could be used. Microtiter plate formats other than the 96-well variety, such as half-volume 96-well or 384-well plates, can be used to increase throughput and/or conserve reagents.

COATING TIME

Coating time depends on binding kinetics, stability, concentration of capture reagent, and incubation temperature. Although different combinations of coating times and temperatures often result in the same coating efficiency, the stability of the capture reagent (which should be determined during method development) influences which conditions to select. Analysts must assess the impact of varying the coating time in order to determine the robustness of the assay procedure.

COATING TEMPERATURE

Coating temperature and time are closely related assay parameters. The coating temperature depends on the binding kinetics and stability of the antigen. Higher temperatures can increase the rate of adsorption and may shorten the coating time, but they are likely to affect interaction sites and to reduce antigen-antibody affinity. Typical combinations of time and temperature are 1–4 h at ambient temperature, 15 min to 2 h at 37°, or overnight at 4°. Analysts should determine the effects of variations in temperature in order to assess the robustness of the assay procedure.

BUFFERS

Buffers used for diluents, coating, blocking, and washing plates can affect overall assay performance. Buffer components can interact with the test sample and inhibit binding. They also can cause low antigen sensitivity or high nonspecific background activity.

Diluent: Buffers [e.g., phosphate-buffered saline (PBS) or imidazole-buffered saline] with polysorbate 20 (0.01%–0.1%) are used commonly for different ELISA steps as a diluent and washing buffer.

Coating buffers: Coating buffers should maximize assay consistency and promote binding of the capture reagent to the solid phase. Commonly used coating buffers include 50 mM carbonate, pH 9.6; 20 mM tris-hydrochloride (tris-HCl), pH 8.5; and 10 mM PBS, pH 7.2. The choice of coating buffer depends on the nature of the individual antigens and should be determined empirically.
Blocking agents and buffers: A blocking agent is a compound (e.g., protein or detergent) that should saturate the remaining immunosorbent binding sites following capture reagent (antibody or antigen) binding. This reduces nonspecific binding of analyte and nonanalyte components to the immunosorbent matrix and/or the absorbed reagent. Nonspecific binding occurs when protein in the test sample binds to the plastic of the microtiter plate or absorbed reagent instead of specifically binding to the capture reagent of interest. Nonspecific binding can be reduced by adding blocking reagent to the wells and by the addition of another protein such as bovine serum albumin (BSA) to the dilution buffer. The choice of blocking agent should be governed by the nature of the capture reagent, plate, coating buffer, test sample diluent, and related factors. If any of these parameters changes, a change in blocking agent may be needed. Commonly used blocking agents include BSA, nonfat milk, gelatin, casein, normal horse serum, fetal bovine serum, polysorbate 20, and others. Several grades of BSA are available commercially, and the optimal grade should be empirically determined for each assay. In addition, many commercial blocking and assay diluent reagents are available for ITM.

Adding Samples and Reagents

Samples and reagents generally are pipetted into the ELISA plate wells. Care should be taken to avoid cross-contamination, frothing, or bubbles. *A sample loading pattern should be included in the test method procedure. For reproducibility and accuracy of results, consistency between the wells of the ELISA plate is very important. This can be achieved by using replicates; however, as mentioned above, care should be taken to avoid replicates in adjacent wells. A common way of avoiding the edge effect is not to use the edge wells at all. Additionally, plate edge effects can also be avoided by reducing assay time, using a low evaporation lid, or sealing the plate with a clear or breathable sterile tape. A (USP 1-Dec-2019) Labor-saving equipment such as electronic pipets, automated liquid handlers, plate washers, and robotic pipets also can be used to improve precision, reduce analyst-to-analyst variability, and increase throughput.

PIPET

Single, multi-channel, and robotic pipets with set or fixed volumes are available. The type and accuracy of pipets should be evaluated for each application. Regular maintenance and professional calibration of pipets should be performed and documented.

PIPET TIPS

A variety of pipet tips are available, some of which are specific to the type of pipet. The type and accuracy of the pipet tip, particularly related to the viscosity and nonspecific binding of the materials, should be evaluated for each application.

Washing

Wash steps are included throughout the ELISA procedure to remove the unbound coating antigen, sample, and detection reagents. Washing is critical for assay performance, can be a source of assay failure, and is important to evaluate during method development. Multiple approaches can be used for washing. Manual procedures include using a squeeze bottle, dipping the microtiter plate in wash buffer, and adding wash buffer with a multi-channel pipet or hand-held multi-channel (8- or 12-pin) manifolds. Analysts should wash carefully to avoid cross-well contamination. Automatic microplate washers generally provide more washing consistency. Strip-well and multi-well washers are available. Most automatic washers can be programmed for different dispensing volumes and speeds, number of washes, speed of buffer aspiration, and amount of residual buffer left in the well. Incorrectly programmed or maintained, as well as incompletely cleaned, automatic washers can cause assay variation and elevated assay background.

Incubation

ELISAs are incubated following the addition of samples and reagents. The optimal time, conditions, and temperature of each incubation step should be determined during method development. Incubation times vary from minutes to overnight. Commonly used incubation temperatures are ambient temperature, 4°, and 37°. ELISA plates commonly are sealed or placed in a secondary container to avoid evaporation or contamination during incubation. Atmospheric conditions such as dry or humidified incubation should be evaluated during method development. Rocking, shaking, or rotating the microtiter plates may be necessary or desirable depending on the kinetics of binding.

Blocking Conditions and Nonspecific Reactions

After immobilization and removal of the unbound antigen or antibody, unoccupied binding sites are blocked to ensure that the measured analyte in the test article or subsequent (detection) reagents does not bind nonspecifically to the solid surface or to the coated antigen or antibody. If nonspecific binding occurs, any reported signal could bias the measurement and may reduce the sensitivity and dynamic range of the assay. Blocking is critical to ensure the sensitivity and/or specificity of the assay. Sources of nonspecific binding fall into two general categories:

1. Ionic or hydrophobic interactions occur when binding is mediated by nonspecific ionic or hydrophobic interactions between assay reagents and the solid surface or another assay reagent.

2. Immunological interactions occur when binding is mediated by unintended antigen–antibody interactions. This occurs when antibody preparations used in the assay interact with other assay reagents. For example, if an ELISA was designed...
to test a serum-derived analyte using murine capture and detection antibodies, antibodies in the test article with reactivity to murine Ig (also known as heterophilic antibodies) could be nonspecifically detected in the assay.

The choice of blocking agent (examples are found in Blocking agents and buffers) is determined empirically, and the balance between the reduction in nonspecific binding and the impact on assay sensitivity should be assessed during method development. Cross-reactivity with other assay reagents should be considered; for example, endogenous biotin is found in milk and serum, and serum may contain antibody to viral or bacterial proteins. Therefore, screening of serum lots may be necessary. The volume of blocking solution added to the well should be greater than the maximum reaction volume used for later steps so that all of the potential surface area that may interfere with the binding reaction is blocked.

In addition, Ig in the test materials can be removed by using buffers that inhibit antibody conformation or aggregate the heterophilic antibodies, by blocking with nonimmune serum, or by removing Fc regions in critical antibody reagents, thereby reducing or eliminating undesired immunological interactions that cannot be addressed by the blocking reagents described above. Negative control wells can be included to monitor nonspecific reactions. The nature of the negative control wells depends on the assay but can include blocked wells without coating antigen, eliminating the primary or secondary antibody, or using buffer in place of sample. Control wells also can be useful as part of system suitability testing.

Pretreatment of Samples

Although ELISA methods are designed to measure an analyte in complex mixtures, the presence of other materials can prove problematic if they interfere with analyte detection. In order to ensure assay specificity, the specific procedure to treat samples to remove nonspecific interfering substances (e.g., reducing agents or precipitates) can be determined empirically during method development and then can be incorporated into the validated assay. Any sample-processing step should be evaluated against the potential that the treatment will alter the test article’s properties and/or introduce further variability that results in biased measurements. Samples, standards, and controls should be prepared and handled in processes as similar to each other as possible. Analysts should verify that sample pretreatments have not damaged the sample so much that it can no longer be measured (e.g., by spiking experiments).

Detector Antibodies

Depending on the ELISA format, detector antibodies labeled with enzyme or other labels can be used as primary or secondary reagents to enable detection of the immobilized analyte. In a direct or competitive ELISA [Figure 2 (A and C)], after the analyte is bound to the immunosorbent surface, excess analyte is washed away and the immobilized analyte is detected using a detector antibody that is considered to be the primary antibody. In other ELISA formats [Figure 2 (B, D, and E)], the analyte-specific Ig (nonconjugated primary antibody) is allowed to bind to the immobilized analyte, and any excess antibody is washed away before the addition of a detector antibody, which is termed the secondary antibody.

To facilitate detection, in all ELISA formats that use enzyme-conjugated antibodies, a substrate specific for the conjugated enzyme is introduced into the assay system. An enzymatic reaction ensues, converting a substrate into a soluble product that can be measured using appropriate wavelengths and a suitable reader.

ELISA sensitivity depends on the quality of the reagents and the detection system, including the label and substrate. If multiple differently conjugated antibodies are available, analysts should select one appropriate for the assay. During this evaluation, the dilution of each conjugate that yields desirable sensitivity and specificity should be determined using appropriate controls.

The most commonly used labeling enzymes for conjugating to antibodies include AP, HRP, and galactosidase. These enzymes are highly specific, sensitive, and stable in catalyzing chromogenic, luminescent, or fluorescent reactions. para-Nitrophenyl phosphate (pNPP) is a commonly used substrate for AP. Commonly used substrates for HRP include TMB, o-phenylenediamine dihydrochloride (OPD), and [2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)] diammonium salt (ABTS) (see Table 2). The substrates for AP and HRP are chromogenic and result in the formation of a colorimetric product that can be measured using a spectrophotometer. Chemiluminescent and fluorescent substrates for AP and HRP also are available, and in many cases they are available as commercial kits. Disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5-chloro)tricyclo[3.3.1.1^2,7]decan}-4-yl) phenyl phosphate (CSPD) is a known chemiluminescent substrate for AP (see Table 2). Well-known fluorescent substrates for galactosidase include 4-methylumbelliferyl galactoside (MG) and nitrophenyl galactoside (NG). If a chemiluminescent substrate is used, then a luminometer is required to quantitate the formed product. A fluorometer is needed if a fluorescent substrate is used in the ELISA.

Table 2 also provides a summary of the advantages and disadvantages of different types of ELISA substrates. Colorimetric substrates have been prevalent since the origin of ELISAs and may yield robust assays that generally are more cost efficient than assays that use chemiluminescent and fluorescent substrates. Nevertheless, chemiluminescent and fluorescent ELISA methods may yield more rapid and sensitive assays with a wider dynamic range than assays that use a colorimetric readout. The final choice of readout should be governed by the assay’s purpose and the requirements of the assay.

### Table 2. Enzyme Conjugates and Substrates

<table>
<thead>
<tr>
<th>Readout</th>
<th>Principle of the Enzymatic Reaction</th>
<th>Enzyme</th>
<th>Substrate</th>
<th>Reader</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colorimetric</td>
<td>Produces a colored product that yields absorbance values directly proportional to analyte concentration</td>
<td>AP, HRP</td>
<td>pNPP, TMB, OPD, ABTS</td>
<td>Spectrophotometer</td>
<td>• Robust</td>
<td>• Less sensitive</td>
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</table>

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### Table 2. Enzyme Conjugates and Substrates (continued)

<table>
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<tr>
<th>Enzyme</th>
<th>Characteristics</th>
<th>Assay System</th>
<th>Equipment</th>
<th>Notes</th>
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</thead>
</table>
| Chemiluminescent | Produces a light emission that is directly proportional to analyte concentration | AP | CSPD | Luminometer | - Wide assay dynamic range  
- Lower coating concentrations  
- More sensitive  
- Rapid signal generation  
- Requires special plates  
- Costly |
| Fluorescent | Produces excitation-induced light emission that is directly proportional to analyte concentration | Galactosidase | MG NG | Fluorometer | - Rapid  
- Sensitive  
- Requires special plates  
- Costly  
- Interference by excipients |

### ASSAY DEVELOPMENT AND VALIDATION PLAN

#### Critical Reagent Development

Key considerations for critical reagents are source, purity, specificity, and stability. For quality measurements, ITMs use reference standards along with critical reagents for analyte capture and detection. Any changes of critical biological reagents should be evaluated (see, for example, guidance contained in (1032)).

#### SOURCE

The availability and quality of the starting material should be controlled so that manufacturing of the (purified) reagent can be reproducibly and consistently performed, potentially over several decades. Because critical reagents are biological molecules, sources can range from chemical synthesis (e.g., peptides) to complex biological matrices (e.g., antibodies prepared from serum, monoclonal antibody from ascites/cell culture, or fermentation/cell culture products). When appropriate for the intended use of the assay, a single lot of a critical reagent can be manufactured to establish a substantial supply and to prevent lot-to-lot variability. In other instances, it may be appropriate to include in the validation multiple lots or multiple suppliers in order to demonstrate that the assay is sufficiently robust for its intended use.

#### PURITY

In general, the purity of critical reagents should be assessed to ensure the removal of impurities and manufacturing process residuals that can influence reagent performance and/or stability.

#### SPECIFICITY

The specificity of a critical reagent refers to its ability to capture or detect only the analyte of interest. The reagent must be specific to the analyte and should show little nonspecific binding or no cross-binding to off-target molecules in complex test materials.

#### STABILITY

The stability of critical reagents should be empirically determined to ensure assay performance over time (issues include accuracy, precision, reproducibility, and assay drift). Long-term (months to years) stability of critical reagents under required storage conditions (e.g., with defined temperatures and containers) should be determined so that appropriate expiry dating can be assigned. Short-term (minutes to days) stability (and freeze/thaw and room temperature stability for frozen critical reagents) also is required to ensure day-to-day assay accuracy, precision, and reproducibility.

#### Feasibility/Pilot Studies

The steps of the process by which an ELISA method is developed, validated, and used in routine sample analysis are described below:

1. Generate or purchase critical reagents to measure the analyte. Determine storage conditions and stability.
2. Understand the performance goals for the assay system.
3. Develop the assay to the point that there is a detectable concentration response curve.
4. Perform method development/robustness testing.
5. Prepare the reference/calibration standard and control and assess stability.
6. Establish assay procedures, appropriate controls and limits, assay and sample acceptance criteria, and instrumentation.
7. Determine method performance, and qualify method for accuracy, specificity, precision, and robustness, including qualification of all applicable sample types to be analyzed.
8. Validate the assay.
9. Implement the method (technology transfer) in the testing laboratory, including training and qualification of analysts.
During assay development, the critical parameters and reagents that are required for the assay should be assessed and set at levels that yield desired assay performance. In many instances several parameters may be evaluated, and well-designed experiments can accelerate assay development, particularly for assessing the potential interaction of several inputs. Many ELISA procedures are product specific, and external reference/calibration standards may not be available. The preparation and stability of reference/calibration standards should be considered early in assay development.

Assay Validation

Assay validation is executed according to guidances from appropriate regulatory bodies [e.g., International Council for Harmonisation (ICH) Q2] to demonstrate that the particular test used for an analyte is appropriate for its intended use. More information about assay validation can be found in Validation of Compendial Procedures (1225) or (1033) if the ELISA is used as a surrogate potency assay. See Appendix 2: Additional Sources of Information about Specific Topics in Validation and Data Analysis.

DATA ANALYSIS

The analysis of ELISA data can be simple (e.g., a linear calibration with inverse regression) or complex (e.g., a nonlinear calibration curve with inverse regression). The type and rigor of data analysis depend largely on the assay system and the intended uses of the assay. For example, data reduction may estimate a concentration (e.g., ng/mL) of an unknown sample using a calibration curve. Other approaches include estimation of the half-maximal inhibitory concentration (IC$_{50}$) or effective concentration (EC$_{50}$), estimation of the amount of a sample that yields the same response as the EC$_{50}$ (or IC$_{50}$) on a standard curve, and an estimate of the relative activity of a test sample compared to a reference/calibration standard. More extensive guidance about statistical methods for potency analysis is given in (1032) and (1034).

In general, ELISA curves are characterized by a nonlinear relationship between the concentration of the analyte of interest and the calculated mean response. Typically, this response curve is defined by a sigmoidal relationship of response to concentration. A wide range of mathematical models can fit standard/calibration curves, and analysts should take care in the selection of an appropriate curve-fitting algorithm. In other cases, ELISA assays are used for qualitative purposes to determine whether a sample is positive or negative based on a sensitivity threshold.

Basic Statistical Analysis

Basic statistical methods are not detailed here. Analytical Data—Interpretation and Treatment (1010) addresses important fundamentals, including data handling; computation of means, standard deviations, and standard errors; detection of and methods to address nonconstant or nonnormal variation; detection of and management of outliers; and procedures for and interpretation of statistical tests and confidence intervals. The concepts behind validation, goals, designs, analysis, and practical methods for validation are described in (1010), (1225), and (1033). Design and Analysis of Biological Assays (111) contains guidance on combining results from independent assays, ±outliers, and confidence intervals. ±(USP 1-Dec-2019)

Nonlinear Statistical Analysis

Nonlinear calibration for immunoassays draws on many sources for statistical design and analysis. These include methods for assessing and addressing nonconstant variance, designs and analysis methods for experiments with complex structures, and validation. The concepts behind linear calibration design, analysis, and inverse regression apply in nonlinear calibration, and professional statisticians can help apply these appropriately.

Reporting Results

Reported estimates of concentration should be understood as having an associated confidence interval based on the results of the validation. The reported value or estimate used to describe a sample can be based on a combined result from multiple assays.

APPENDICES

Appendix 1: Abbreviations

Ab: Antibody
ABTS: 2,2'-Azino-bis[3-ethyl-benzothiazoline-6-sulfonic acid]diammonium salt
Ag: Antigen
Anti-Ig: Anti-immunoglobulin
AP: Alkaline phosphatase
BSA: Bovine serum albumin
CSPD: Disodium 3-(4-methoxyspiro[1,2-dioxetane-3,2'-5'-(5'-chloro)tricyclo[3.3.1.1^{3,7}]decan]-4-yl)phenyl phosphate

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Not official text. Please refer to the currently official version of the applicable USP–NF or FCC standard for compliance purposes.
EIA: Enzyme immunoassay
ELISA: Enzyme-linked immunosorbent assay
HRP: Horseradish peroxidase
Ig: Immunoglobulin
ITM: Immunological test method
MG: 4-Methylumbelliferyl galactoside
NG: Nitrophenyl galactoside
OPD: o-Phenylenediamine dihydrochloride
PBS: Phosphate-buffered saline
pNPP: para-Nitrophenyl phosphate
PVDF: Polyvinylidene fluoride
TMB: 3,3′,5,5′-Tetramethylbenzidine

Appendix 2: Additional Sources of Information about Specific Topics in Validation and Data Analysis

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<th>Means</th>
<th>X</th>
<th>Design and Analysis of Biological Assays (111)</th>
<th>Validation of Compendial Procedures (1225)</th>
<th>Biological Assay Chapters (1032), (1033), and (1034)</th>
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<td>Standard errors</td>
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<td>Combining results from multiple assays</td>
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〈1104〉 IMMUNOLOGICAL TEST METHODS—IMMUNOBLOT ANALYSIS

INTRODUCTION

Definition and Scope

The chapter is part of a group of general information chapters for immunological test methods (Immunological Test Methods—General Considerations (1102), Immunological Test Methods—Enzyme-Linked Immunosorbent Assay (ELISA) (1103), and Immunological Test Methods—Surface Plasmon Resonance (1105)) that provides analysts with general information about principles, method development, method validation, and data evaluation for immunoblot analysis. Immunoblot analysis is defined as any method in which an antibody is used for detection of one or more analytes (e.g., proteins, polysaccharides) that has been transferred to a test membrane surface. Immunoblot methods are typically classified by whether electrophoretic separation occurs as a part of the immunoblot procedure. Electrophoretic separation is based on molecular weights and charge differences of a population of molecules. See Capillary Electrophoresis (1053), Biotechnology-Derived Articles—Isoelectric Focusing (1054), and Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis (1056) for a detailed description of electrophoretic separation methods. An example of immunoblot analysis involving electrophoretic separation is the Western blot, which was first described in the scientific literature in the late 1970s. Another approach for immunoblot analysis is to perform molecule detection using an antibody without prior electrophoretic separation. Examples of this nonelectrophoretic type of approach are the slot or dot blot (slot/dot).

The scope of this chapter includes only those methods in which an antibody is used for the detection of a molecule bound to a membrane. Therefore this chapter does not discuss procedures that use nonimmunological means of detection.

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ASSAY SELECTION

Nonelectrophoretic Assay (Slot/Dot Blot)

The slot/dot blot method is a simplified, nonelectrophoretic method in which a mixture containing the analyte(s) for detection is first applied directly to the membrane using a vacuum manifold machined to contain regularly spaced rectangular slots. The slot/dot blot method is faster and simpler because there is no electrophoretic separation of the multiple, individual analytes that may be present in the mixture. For these reasons, it can be readily adapted for automated analysis of multiple samples, for which a number of systems are commercially available, but it offers no information about the molecular weight and only limited information regarding the quantity of sample. Although it can be set up in a quantitative format, the method usually is used to produce a qualitative result, e.g., confirming identity by demonstrating the presence or absence of specific antigens by means of an immunocomplex detection system. After the analytes are bound to the membrane and unbound sites are blocked, analysts use a detector antibody to determine the presence or absence of the analyte or analytes of interest. The uniform shape of the slot blot and its greater surface area for analyte binding make it better suited than the dot blot for quantitative applications and analysis by densitometry.

Electrophoretic Assay

Electrophoretic blotting methods (commonly called Western blots) are widely used for analyzing mixtures of proteins. The Western blot is a powerful tool to study the identification, relative concentration, relative molecular weight, and posttranslational modifications of specific proteins. In Western blots, the proteins of the sample are separated using gel electrophoresis. Protein separation may be based on molecular weight alone or on isoelectric point (pl) and molecular weight. Proteins migrate either in one dimension (1D) or in two dimensions (2D) through a gel. When proteins are separated by their molecular weights, the smaller proteins migrate faster and separate according to molecular weight. When analysts use a 2D gel, proteins are separated according to pl in the first dimension, and then according to their molecular weights in the second dimension. After separation, the proteins are transferred to a membrane, the membrane is blocked to avoid nonspecific binding of subsequent assay reagents, and the protein of interest is detected using specific antibodies.

A bound antibody can be detected by different methods, including colorimetric detection, fluorescent detection, chemiluminescent detection, and radioactive detection. Upon detection of the protein(s) of interest, immunoblot quantitation can be indirectly performed by densitometry.

1D ELECTROPHORESIS

In 1D electrophoresis, individual proteins or groups of proteins are separated by molecular weight for further analysis by Western blot. Using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), proteins migrate in response to differences in electrical charge through a 3D network of fibers and pores. The network is formed as the bifunctional bisacrylamide, or other cross-linker, cross-links adjacent polyacrylamide chains to form a gel (see also USP general chapter (1056)). The combination of gel pore size and protein characteristics determines the migration of proteins. Separated proteins are detected subsequently by Western blot analysis using antibodies specific to the target proteins. By means of Western blot analysis, a test sample can be compared to a standard, and the appearance of degradants and impurities specifically related to the target proteins can be monitored if the detection antibody can still recognize the altered forms of the protein. Although a high level of sensitivity can be achieved by this approach, separation of individual proteins at similar molecular weights may not be achieved. If analysts must probe individual proteins at similar molecular weights, 2D separations may be required.

2D ELECTROPHORESIS

In 2D electrophoresis, individual proteins or groups of proteins are separated in the first dimension by isoelectric focusing (IEF; charge) and in the second dimension by electrophoresis in the presence of SDS (molecular weight). Separating proteins this way allows information to be obtained not only about molecular weight, as in 1D gels, but also about the charge of a protein. Two-dimensional gels are a useful choice for resolving complex mixtures and for assessing protein antibody specificity (e.g., evaluation of host cell proteins).

Membrane, Reagent, and Detection Options

MEMBRANES

Generally, both nitrocellulose and polyvinylidene fluoride (PVDF) membranes are used for immunoblot methods. For cost considerations, nitrocellulose membranes are often preferred over PVDF membranes for slot/dot blots (or vacuum blotting), but due to their greater mechanical strength, PVDF membranes should be considered if stripping and reprobing are required.

BLOCKING REAGENTS

Following transfer or binding of protein to membranes, the unoccupied binding sites on the membranes must be blocked to prevent nonspecific binding of subsequent reagents. Most detection probes are proteins that also can bind to the membrane. Failure to appropriately block the membrane sites can result in nonspecific binding and high background. A number of blocking reagents are available, including gelatin, nonfat milk, and bovine serum albumin (BSA). Proteins should be unrelated to the antigens used in the study. Because these reagents often have lot-to-lot variability, they may require qualification. They must be evaluated with the detection system selected and optimized using that detection system for minimal background with no
Once an immunocomplex containing the enzyme–conjugate reagent has formed, analysts used for detection. If the secondary antibody is biotinylated, biotin–avidin–HRP or –AP complexes can be used for detection.

**METHODS OF DETECTION**

Immunological detection of analytes in any type of immunoblot can be direct or indirect. The choice of format depends on a combination of the level of sensitivity required and the quality of the antisera available. For identity or product detection, sensitivity usually is not critical, and direct detection via a conjugated antibody is commonly used, which often simplifies and shortens the time required to execute the method. Alternatively, indirect detection, usually by the use of a conjugated anti-species reagent, can be used to improve sensitivity. On some occasions, the analyte being detected is actually an antibody, as in the case of a monoclonal antibody that is being developed as a drug. In this case, antibodies specific for the antibody (e.g., anti-idiotypic antibodies) can be used.

**Primary antibody:** The primary antibody is selected based on its specificity for the analyte or protein. Although polyclonal anti-sera can offer a broad range of detection against a potentially large set of epitopes, an unwanted cross-reaction resulting in decreased specificity may occur. If this cannot be overcome by assay optimization, a monoclonal antibody or groups of selected monoclonal antibodies can be used. Monoclonal antibodies are often advantageous for long-term studies, because they yield a consistent supply of antibody against a specific epitope. The use of monoclonal antibodies directly limits the number of epitopes involved in the detection of the target. This must be evaluated for each application. The primary antibody may be directly conjugated or used in conjunction with a secondary antibody and an appropriate detection system. The optimal antibody concentration usually is considered to be the greatest dilution of antibody that results in a strong positive signal with minimal background. This must be optimized in conjunction with the block and detection system selected. The primary antibody should be qualified before assay use.

**Secondary antibody:** The secondary antibody typically is directed against the species of the primary antibody immunoglobulin (which is specific for the analyte, e.g., goat anti-mouse IgG). Enzymes such as horseradish peroxidase (HRP) or alkaline phosphatase (AP) typically are linked to the secondary antibody, but other labels such as fluorophores or gold particles can be used for detection. If the secondary antibody is biotinylated, biotin–avidin–HRP or –AP complexes can be used for detection.

**Detection enzyme and substrate:** Once an immunocomplex containing the enzyme–conjugate reagent has formed, analysts add a suitable substrate to the assay. This reaction results in production of a colored precipitate or a fluorescent or chemiluminescent product that can be recorded, measured, and analyzed further. A broad range of detection options is available to best fit individual applications and intended uses. A number of these are described in (1103) and (1102), as well as in Table 1.

<table>
<thead>
<tr>
<th>Readout</th>
<th>Principle of the Enzymatic Reaction</th>
<th>Enzyme</th>
<th>Substrate</th>
<th>Detection</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colorimetric</td>
<td>Produces a colored product that yields absorbance values directly proportional to analyte concentration</td>
<td>AP&lt;sup&gt;a&lt;/sup&gt;</td>
<td>pNPP&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Spectrophotometer</td>
<td>— Robust — Economical — Reagent availability</td>
<td>— Time-consuming — Less sensitive than other methods</td>
</tr>
<tr>
<td>Chemiluminescent</td>
<td>Produces a light emission that is directly proportional to analyte concentration</td>
<td>AP, HRP</td>
<td>CSPD&lt;sup&gt;f&lt;/sup&gt;</td>
<td>Luminometer, photographic film (CCD camera)</td>
<td>— Wide assay dynamic range — Very sensitive — Rapid signal generation</td>
<td>— Reproducibility can be challenging</td>
</tr>
<tr>
<td>Fluorescent</td>
<td>Produces excitation-induced light emission that is directly proportional to analyte concentration</td>
<td>Galactosidase, fluorescently labeled antibody</td>
<td>M&lt;sub&gt;c&lt;/sub&gt;</td>
<td>Fluorometer (CCD camera with filters)</td>
<td>— Rapid — Sensitive</td>
<td>— Interference by excipients</td>
</tr>
<tr>
<td>Radioactive</td>
<td>Antigen is labeled with a radioactive isotope. Radiation is proportional to analyte concentration.</td>
<td>—</td>
<td>—</td>
<td>Scintillation counter</td>
<td>— Easy to quantitate — Rapid</td>
<td>— Safety risk with exposure — Radioactive waste</td>
</tr>
</tbody>
</table>

<sup>a</sup> Alkaline phosphatase.<br>
<sup>b</sup> Horseradish peroxidase.<br>
<sup>c</sup> 4-Methylumbelliferyl galactoside.<br>
<sup>d</sup> 3,3',5,5'-Tetramethylbenzidine.<br>
<sup>e</sup> o-Phenylenediamine dihydrochloride.<br>
<sup>f</sup> 2,2'-Azoxy-bis[3-ethyl-benzo(thiazolone-6-sulfonic acid)]diammonium salt.<br>
<sup>g</sup> Disodium 3-(4-methoxyphosphor(1,2-dioxetane-3,2'-((5'-chioro)tricyclo[3.3.1.1<sup>7,11</sup>decan-4-yl)phenyl phosphate.<br>
<sup>h</sup> Charge-coupled device.<br>
<sup>i</sup> Nitrophenyl galactoside.

**METHOD DEVELOPMENT**

Method development can proceed on the basis of the background information just provided. The scope of method development, and eventually method validation, are dictated by the purpose of the method. The purpose determines the format.
for the assay and other requirements for the test, and therefore, the purpose should be determined first. The following sections explore considerations for each method’s purpose.

**Intended Purpose of the Method**

**IDENTITY TESTING**

In the case of identity tests, analysts want to detect the presence of a protein; therefore, demonstration of specificity is essential and required. For this purpose, analysts also control the quantity of protein in the sample. Thus, the limits of detection (LOD), limits of quantitation (LOQ), and other measures of quantity are not required attributes of the method. Examples include material identity assays that demonstrate the isotype of an IgG and, in some cases, demonstrate the specificity of an antibody in a method validation. If there is no interference from the matrix or potential cross-reaction with other materials present in the sample, then a simple slot/dot blot may suffice. If multiple proteins in the sample display immunoreactivity and must be distinguished from each other, another separation procedure must be used before blotting and immunostaining. The complexity of the proteins in the sample and the usefulness of the additional information gained using an electrophoretic separation help determine if a slot/dot blot can meet the needs of the test.

**LIMIT TESTING**

In other applications, analysts may want to show that an impurity has been removed to a level below toxicological concern. In many cases, a limit test is used when it is possible to say yes or no about the presence or absence of a protein below a predetermined level. This simplifies the development and validation of the method. With densitometry (scanning or imaging) equipment, the intensity of spots or bands can be determined relative to a standard curve, resulting in an estimate of concentration. An LOD should be determined to establish the appropriate limit threshold for the method. A dot blot may be suitable for either circumstance if the specificity of the antibody in the sample matrix can be demonstrated.

Another common purpose for an immunoblot is to show the presence or absence of a protein expressed from a culture. In this situation, analysts want to establish the identity of the protein by immunostaining, as well as verify that the protein has the expected molecular weight. This provides further assurance of no nonspecific interactions with other proteins in a complex matrix that generates the signal in the blot.

**SPECIFICITY TESTING**

Characterizing the specificity of the reagents for an ELISA impurity test or an immunoaffinity column also is a common immunoblot purpose. This is another form of an identity test in which the desired endpoint is demonstration of the specificity of binding between the antigen and the antibody. The result of the measurement is a demonstration of binding to a select group of the total protein population in the sample or binding to the whole population of proteins in the sample, as required for host-cell protein assays. In order to demonstrate the specificity of an antibody relative to a population of proteins, analysts typically must carry out electrophoretic or other separations. Showing, by means of immunostaining, that a protein of the right molecular weight or pI can be recognized by the antibody is a powerful demonstration both of specificity toward a given protein and the absence of binding to other proteins. In addition, having, within the same experiment, the appropriate positive control samples that are known to contain the protein, and the appropriate negative samples that are known not to contain the protein, makes a convincing argument for the specificity and selectivity of the antibody when analysts validate an ELISA method for protein impurities. Electrophoretic separations can be done in one dimension using either SDS–PAGE (for molecular weight) or isoelectric focusing (IEF; for isoelectric point) for a limited number of proteins with known molecular weights. Electrophoretic separations also can be done in two dimensions (e.g., IEF followed by SDS–PAGE) to show selectivity and specificity toward a more heterogeneous population of proteins. A 2D Western blot commonly is used to demonstrate the specificity of a polyclonal antibody candidate directed against a host-cell protein (HCP) antigen preparation before development of a quantitative ELISA for that purpose.

**Assay Mode and Sample Introduction**

After considering the critical elements required for each purpose of the method, the analyst can use this information to select the most appropriate assay mode. The main points to consider when developing an immunoblot method are shown in Figure 1. If appropriate, spotting samples on a membrane or applying by vacuum is the easiest and most convenient way to introduce a sample to an immunoblot membrane. However, low levels of nonspecific binding from multiple proteins can create additive nonspecific interference in dot blots or slot blots, resulting in background levels that appear to be the desired analyte.
Electrophoretic separations, although time consuming, can be useful for separating and further distinguishing specific and nonspecific binding. Analysts must trade sensitivity for selectivity in going from a single dimension to two dimensions because of the further separation of immunoreactive species from a single band into multiple spots, as is the case with the heterogeneity seen in sialylated proteins or deamidated species.

### Assay Controls and Standards

Controls and standards are selected based on the purpose of the assay and the information needed during development. Protein molecular weight markers can be used to obtain an accurate estimate of the molecular weight of immunoreactive species. The use of positive and negative controls is helpful for troubleshooting throughout the experimental design process. Standards or positive and negative controls can be used to assess system suitability and to establish method performance. A positive control can confirm appropriate protein migration and can confirm that membrane transfer has reached completion. A negative control is useful for assessing nonspecific interactions. A method sensitivity control near the LOQ can be used to measure the consistency of the method near the LOQ to evaluate changes in assay performance.

### Membrane Selection

A membrane is selected based on the application and the protein being measured. Membranes with various pore sizes should be available and should be suitable for the molecular weight of the protein of interest to aid in appropriate transfer of different sizes of proteins. If a chemical staining method is known to work on a specific membrane with a specific protein, then it is advantageous to show that the protein binds to the membrane and is stained before analysts work on the immunostaining.

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**Figure 1. Method Development Flow Chart.**

| Decide Assay Mode/Sample Introduction | • Slot/dot blot  
| • 1D separation  
| • 2D separation |  
| Assay Controls/Standards | • Standards  
| • Negative controls  
| • Sensitivity controls |  
| Membrane Selection | • Nitrocellulose  
| • PVDF  
| • Show binding of the antigen to the membrane with a chemical stain |  
| Optimize Blot Transfer | • Transfer time (gel electrophoresis)  
| • Vacuum time  
| • Spot volume |  
| Antibody Specificity | • Primary antibody  
| • Secondary or detection antibody matched to the primary antibody  
| • Secondary or detection antibody matched to the selected substrate/detection system  
| • Selected matrix spikes and controls |  
| Select Blocking Reagent | • Absence of antigen analyte  
| • Blocks nonspecific binding of antibody reagents and secondary detection system |  
| Titrate Primary and Secondary Antibodies | • Primary antibody  
| • Secondary antibody  
| • Simultaneous titration with matrix design |  
| Substrate Incubation and Data Acquisition | • Substrate incubation time  
| • Data acquisition time  
| • Data analysis |
steps for the assay. Electrophoretic separations followed by transfer to a membrane should be optimized with chemical staining, e.g., with sensitive fluorescent stains or silver stains and at potentially higher loads before analysts work on lower load levels required for blot optimization. Stains such as Coomassie or colloidal Coomassie may not have sufficient sensitivity to detect a low level of impurities required for certain applications.

**Optimize Blot Transfer**

Analysts should optimize transfer times from the gel to the membrane. Larger proteins require more time for transfer than smaller proteins. Small proteins may be lost during long transfer times and can transfer from the gel all the way through the membrane and be lost on the other side. The density of the gel and gradient gels can result in nonuniformity of transfer from the top to the bottom of the gel. During transfer optimization, many method developers use multiple membranes in order to capture proteins that transfer through the first membrane. Chemical staining of both the gel and the membranes can provide useful information about the location of the proteins transferred from the gel to support, either extending or reducing the transfer time.

After they select the assay mode, analysts next investigate spotting of the antigen or transfer from a gel to the appropriate membrane at various relevant concentration levels. Levels of analyte above the concentration needed for a Western blot may be required at first to determine if transfer and recognition by the antibodies is possible. If the analyte is present in low concentrations, spiking may be necessary to show its location during transfer optimization. Because of the potential variability of immunostaining and transfer, a sensitivity control or several levels of controls should be incorporated into the method based on the analyte titration above the background level. This can be adjusted as method development progresses.

**Antibody Specificity**

Analysts should demonstrate antibody specificity early in immunoblot method development. If possible, they should test samples of the matrix without the analyte and should show an absence of response. In contrast, samples that contain analyte spiked into the matrix should show a positive response, demonstrating the specificity of the antibodies.

Analysts also should demonstrate the specificity of the secondary antibody conjugate or label. Control immunoblots with lanes or spots of primary antibody and control matrix samples containing the analyte as a negative control can show that the secondary antibody is binding to the primary antibody and not to proteins found in the matrix. Commercial sources for enzyme conjugates or fluorescent-labeled anti-species antibodies are readily available and normally are screened or affinity purified against the species of antibody being detected, which eliminates some of the early work needed to achieve the desired specificity. The secondary antibody or detection system must be matched with the detection equipment and the desired sensitivity of the assay, e.g., fluorescence, colorimetric precipitating substrates, or chemiluminescence.

**Select Blocking Reagent**

Replicate membranes can be blocked with previously described blocking agents as analysts select the most appropriate blocking reagent and the amount of time required to minimize background by means of subsequent primary and secondary antibody incubations. Analytes titrated at multiple concentrations on the membrane allow analysts to assess the amount of signal to the amount of noise (background) with various blocking reagents followed by immunostaining with the primary antibody, labeled secondary antibody, and substrate, if needed, for visualization. This titration also serves as the starting point for examining LOD and LOQ for limit tests and quantitative measurements. The LOD for immunoblots is determined by the level of nonspecific background relative to the specific signal from the analyte. As is the case with any other analytical method, if the background and signal are equal, there is no distinction between the signal and the noise.

**Titrate Primary and Secondary Antibodies**

Titrating the level of primary and secondary antibody from low to high dilutions can also, as with a blocking reagent, be used to select an antibody concentration that reduces background binding in the blank regions surrounding protein spots or bands, and can optimize the signal from the analyte. A matrix grid that varies the level of primary signal against secondary signal can be useful for optimizing the background, improving analyte signal, and reducing the consumption requirements for expensive antibody reagents.

Imunoaffinity chromatography against a highly purified antigen can be used to reduce the level of nonspecific interference for all of the immunological reagents used in an immunoblot. The method developer must be cautious that the selectivity, specificity, and affinity of the primary antibody are not lost in affinity purification because of high-affinity antibodies that remain on the antigen column or because of the destruction of antibody binding caused by elution conditions. For the secondary antibody, immunoaffinity-purified anti-species antibodies are available commercially with a variety of possible labels conjugated to the antibody.

**Substrate Incubation and Data Acquisition**

Analysts can optimize substrate development time for enzymes in order to minimize background and improve the LOD and LOQ. Excessive substrate development times for precipitating substrates can result in an intensification of the background level relative to the specific signal from the desired analyte. If the blot is agitated during substrate incubation, undesired swirling patterns of product from precipitating substrates can form. Too short an incubation time results in a less-specific signal, but too long a time can result in high background and poor resolution. Most enzyme conjugates have an optimum development time. Fluorescent labels and chemiluminescent labels have the advantage of acquisition by scanning instrumentation that can store...
data electronically and perhaps acquire image signals in an additive manner. Fluorescent labels have the added advantages that
the signal is stable with time, numerous experiments for development time can be obtained with a single blot, and optimization
of signal acquisition can be performed on a single blot.

**PROCEDURES**

**Slot/Dot Blots**

Using an appropriate slot/dot apparatus, analysts can make antigens of interest adhere to a suitable membrane (e.g.,
nitrocellulose) by gravity or vacuum filtration, followed by addition and incubation of antigen-specific antibodies that bind to
epitopes on the antigens. Remaining binding sites on the membrane are blocked by the addition of nonspecific antigen (e.g.,
BSA), followed by probing of the antigen-specific antibodies with a detection system [e.g., protein A/G conjugated to HRP binds
to the antibodies that then are visualized using a 4-chloro-naphthol (4-CN) peroxidase substrate]. Positive identification is the
development of dots or bands on the membrane. A negative result remains white or exhibits faint bands that are considerably
lighter than positive bands.

**1D Immunoblotting**

**PREPARATION OF SDS–PAGE GELS**

Analysts should choose an SDS–PAGE gel with a content of acrylamide–bisacrylamide suitable for the molecular weight(s) of
the protein(s) of interest; i.e., the smaller the molecular weight of the protein, the higher the percentage of mono- or
bisacrylamide, and conversely, the larger the molecular weight of the protein, the lower the percentage of mono- or
bisacrylamide.

Uniform-concentration gels have separation ranges as shown in Table 2, and gradient gels have a separation range as shown
in Table 3. Gels can be purchased ready-made or can be produced in the laboratory according to procedures in (1056).

<table>
<thead>
<tr>
<th>Acrylamide Concentration (%)</th>
<th>Linear Range of Separation (kD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>57–212</td>
</tr>
<tr>
<td>7.5</td>
<td>36–94</td>
</tr>
<tr>
<td>10</td>
<td>20–80</td>
</tr>
<tr>
<td>12</td>
<td>12–60</td>
</tr>
<tr>
<td>15</td>
<td>10–43</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Acrylamide (%)</th>
<th>Protein Range (kD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5–15</td>
<td>20–250</td>
</tr>
<tr>
<td>5–20</td>
<td>10–200</td>
</tr>
<tr>
<td>10–20</td>
<td>10–150</td>
</tr>
<tr>
<td>8–20</td>
<td>8–150</td>
</tr>
</tbody>
</table>

**SAMPLES AND STANDARD**

To prepare samples, analysts typically must lyse cells and tissues in order to release the proteins of interest. The main
consideration when choosing a lysis buffer is whether the antibody chosen for detection of the protein(s) of interest can
recognize denatured samples. When this is not the case, analysts use buffers without detergent or with relatively mild, nonionic
detergent.

Samples should be treated (e.g., reduced, nonreduced, or denatured) according to general chapter (1056), and when a
sample of unknown protein content is used, a series of dilutions should be loaded onto the gel. Standards (molecular weight
markers) should be treated according to the manufacturer’s instructions.

**ELECTROPHORESIS**

Before applying samples to the stacking gel wells according to (1056), analysts denature samples (e.g., heat at 95°–100° for
5 min). An appropriate volume of sample is loaded onto the gel, and a voltage of 8 V/cm applied until the dye has moved into
the resolving gel. Afterward, the voltage is increased to 15 V/cm, and the separation is run until the bromophenol blue reaches
the bottom. If a commercially available gel is used, the manufacturer’s recommendations are followed. Table 4 shows common
sample-loading volumes for particular gels.
Table 4. Common Sample-Loading Volumes

<table>
<thead>
<tr>
<th>Wells</th>
<th>Gel Thickness (mm)</th>
<th>Maximum Sample Load Volume (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>1.0</td>
<td>25</td>
</tr>
<tr>
<td>10</td>
<td>1.5</td>
<td>37</td>
</tr>
<tr>
<td>12</td>
<td>1.0</td>
<td>20</td>
</tr>
<tr>
<td>15</td>
<td>1.0</td>
<td>15</td>
</tr>
<tr>
<td>15</td>
<td>1.5</td>
<td>25</td>
</tr>
</tbody>
</table>

TRANSFER

After electrophoresis, the proteins of interest can be blotted to a membrane such as nitrocellulose or PVDF with a pore size that is appropriate for the molecular weight of the proteins of interest. Both nitrocellulose and PVDF have a protein-binding capacity of about 100–200 µg/cm². PVDF is more chemically resistant than nitrocellulose and is easier to handle. Detailed instructions for the transfer process can be found on the websites of the manufacturers of transfer apparatus and vary depending on the system.

Transfer can be done in wet or semi-dry conditions. Semi-dry transfer generally is faster, but wet transfer is especially recommended for large proteins >100 kD. For both kinds of transfer, the membrane is placed next to the gel. The two are sandwiched between absorbent materials, and the sandwich is clamped between solid supports to maintain tight contact between the gel and membrane.

A standard buffer for transfer is the same as the buffer used for the migration or running buffer without SDS, but with the addition of methanol to a final concentration of 20%. For proteins larger than 80 kD, SDS should be included at a final concentration of 0.1%. Lowering methanol in the transfer buffer also promotes swelling of the gel, allowing large proteins to transfer more easily. Table 5 contains common buffers used for Western blot methods.

Table 5. Common Western Blot Buffer Formulations

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Content</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample buffer 2× (nonreducing)</td>
<td>1.89 g of Tris 5.0 g of SDS 50 mg of bromophenol blue 25.0 mL of glycerol</td>
</tr>
<tr>
<td>(1D electrophoresis)</td>
<td>Adjust with HCl to a pH of 6.8. Add water to 125 mL.</td>
</tr>
<tr>
<td>Sample buffer 2× (reducing)</td>
<td>To nonreducing sample buffer: Add 12.5 mL of 2-mercaptoethanol before adjusting the pH. Alternatively, use 1.93 g of Tris, and add a suitable quantity of DTT* to obtain a final concentration of 100 mM DTT.</td>
</tr>
<tr>
<td>(1D electrophoresis)</td>
<td>151.4 g of Tris 721.0 g of glycine 50.0 g of SDS 500 mL of water Add water to 5000 mL. Adjust to a pH of 8.1–8.8.</td>
</tr>
<tr>
<td>Running buffer 10× (1D electrophoresis)</td>
<td>151.4 g of Tris 721.0 g of glycine Add water to 5000 mL. Adjust to a pH of 8.1–8.8.</td>
</tr>
<tr>
<td>Transfer buffer 10×</td>
<td>100 mL of 10× stock 500 mL of water 200 mL of methanol Add water to 1000 mL.</td>
</tr>
<tr>
<td>TBS 10×</td>
<td>24.23 g of Tris base 80.06 g of NaCl Mix in 800 mL of ultrapure water. Adjust with pure HCl to a pH of 7.6. Add water to 1000 mL.</td>
</tr>
<tr>
<td>TBS-T</td>
<td>100 mL of TBS 10× 900 mL of water 1 mL of polysorbate 20</td>
</tr>
<tr>
<td>8.5 M urea stock</td>
<td>510 g of urea Add water to 1000 mL.</td>
</tr>
</tbody>
</table>

*Not official text. Please refer to the currently official version of the applicable USP–NF or FCC standard for compliance purposes.

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Methanol is necessary only if analysts use nitrocellulose. If they use PVDF, they can remove methanol from the transfer buffer and need only to activate the PVDF before they assemble the gel and membrane sandwich.

In semidy transfer, a sandwich of paper/gel/membrane/paper wetted in transfer buffer is placed directly between the cathode and anode. During wet transfer the membrane should be closest to the positive electrode, and the gel should be closest to the negative electrode. The composition of the transfer buffer is not necessarily the same as the migration or running buffer. Analysts should consult the apparatus manufacturer’s protocol, and it is common to add both SDS and methanol. The balance of SDS and methanol in the transfer buffer, the proteins’ molecular weights, and the gel percentage can affect transfer efficiency for both wet and semidy transfers.

**BLOCKING**

Blocking the membrane prevents nonspecific background binding of the primary and secondary antibodies to the membrane. Traditionally, one of two blocking solutions is used: nonfat milk or BSA (Cohn fraction V). Milk is cheaper but is not recommended for studies of phosphoproteins. To prepare a 5% milk or BSA solution, weigh 5 g/100 mL of Tris-buffered saline containing polysorbate 20 buffer (TBS-T; see Table 5). Mix well, and filter. Failure to filter can lead to spotting in which tiny dark grains contaminate the blot during development. Incubate at 4° for 1 h with gentle shaking. Rinse in TBS-T after the incubation.

**PRIMARY ANTIBODY AND INCUBATION BUFFER**

Dilute the antibody with blocking buffer at a proper dilution (1:100–1:3000, depending on antibody titer), and optimize the dilution according to the results. Too much antibody can result in nonspecific bands.

**INCUBATION TIME**

Incubation time can vary between a few hours and overnight, and depends on the binding affinity of the antibody for the protein and the abundance of protein. A more dilute antibody with a prolonged incubation may improve specific binding.

**INCUBATION TEMPERATURE**

It is best to incubate under cold temperatures. When analysts incubate in blocking buffer overnight, they should incubate at 4° to prevent contamination from bacterial growth, and should gently agitate the antibody solution to enable adequate homogeneous covering of the membrane.

**SECONDARY ANTIBODY AND INCUBATION BUFFER**

Handle the secondary antibody and incubation buffer as follows. Wash the membrane several times in TBS-T while agitating to remove residual primary antibody. Dilute the secondary antibody with TBS-T at the suggested dilution. Too much secondary antibody can result in nonspecific bands. Incubate the blot at room temperature for 1–2 h with gentle agitation. Table 7 shows multiple options for secondary detection reagents and methods. More details are available in the Immunoblot Data Analysis section below.

**Slot/Dot Blot**

The procedure is similar to the procedure for 1D immunoblotting, but differs because protein samples are not separated electrophoretically but are spotted directly onto the membrane either manually or by use of a blotting unit (dot or slot blot format).

**PROCEDURE USING MANUAL SPOTTING**

Handle manual spotting as follows. Place a dry filter paper on a stack of dry paper towels. Place filter paper that is pre-wet with transfer buffer on top of the dry filter paper. Place a pre-wet membrane on top of the pre-wet filter paper. Samples are spotted onto the pre-wet membrane and are allowed to absorb into the membrane. After the sample is absorbed, place the membrane on a clean, dry filter paper to dry.

**PROCEDURE USING A VACUUM-BLOTTING UNIT**

Analysts typically use a vacuum-blotting unit as follows. Prepare a membrane, and place it in the blotting unit according to the manufacturer’s instructions. Apply vacuum to the blotting unit to remove excess buffer. To improve solubility, dissolve the

---

**Table 5. Common Western Blot Buffer Formulations (continued)**

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Content</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample buffer</td>
<td>47 mL of 8.5 M urea stock</td>
</tr>
<tr>
<td>2D electrophoresis</td>
<td>385 mg of tributyl phosphine (TBP)</td>
</tr>
<tr>
<td></td>
<td>2 g of CHAPS</td>
</tr>
<tr>
<td></td>
<td>25 mg of bromophenol blue</td>
</tr>
<tr>
<td></td>
<td>1% carrier ampholytes of choice</td>
</tr>
</tbody>
</table>

*a* Dithiothreitol.<br>
*b* 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate.
sample in a buffer, and if it is not clear, remove precipitates by centrifugation. If the sample is too viscous to pipet, then dilute it further with buffer. With the vacuum off, carefully pipet samples into the wells, and apply vacuum to the blotting unit. After all the samples have filtered through the membrane, turn off the vacuum, add buffer to each well to wash down the sides, and apply vacuum again. Remove the membrane, and proceed with immunoblotting.

**2D Immunoblotting**

**SAMPLE PREPARATION**

The compounds used to solubilize proteins must not increase the ionic strength of the solution. For example, a common sample solubilization solution is the following: 8 M urea, 50 mM dithiothreitol (DTT) or 2 mM tributyl phosphine (TBP), 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 0.2% carrier ampholytes, and 0.0002% bromophenol blue. The addition of carrier ampholytes enhances the solubility of proteins as they approach their isoelectric points. The use of ampholytes produces an approximately uniform conductivity across the pH gradient without affecting its shape, meaning that the concentration of carrier ampholytes should be optimized.

**CHARGE SEPARATION**

Several vendors produce and sell immobilized pH gradient (IPG) strips, or they can be made in-house according to (1054). The choice of IPG strips depends on the pI of the proteins of interest. The size of the IPG should match the size of the second-dimension gel. The amount of protein in each sample should be determined, and the amounts loaded on the IPG strips should be in the range of 10–300 µg, depending on the size of the IPG. The sample and standard should be loaded according to the manufacturer’s instructions or according to (1054). Analysts then proceed with the isoelectric focusing by applying the electrical parameters described in (1054) or by the manufacturer.

**MOLECULAR WEIGHT SEPARATION**

After charge separation, analysts must equilibrate the strip in SDS-containing buffer before separation in the second dimension to determine molecular weight (as described previously in the **1D Immunoblotting** section). Analysts should position the strip directly on top of the gel, then secure the strip by overlaying it with 0.5%–1.0% agarose prepared in SDS–PAGE running buffer. To track the ion front in the second dimension, analysts can add bromophenol blue to the agarose.

**IMMUNOBLOT DATA ANALYSIS**

The presence or absence of bands usually is determined by comparison to a control (highly characterized antigens known or qualified to give a precise or expected response) of a type that is similar to the antigen being processed. Although analysts usually perform a qualitative comparison, bands or dots can be quantitated using a detection system (e.g., after incubating in a solution containing 4-CN peroxidase substrate; also see Table 1), and are compared to the control bands run in parallel (e.g., in the same gel).

**Detection Options**

**ENHANCED CHEMILUMINESCENCE**

Enhanced chemiluminescence is a popular method for detection in immunoblot analysis because it is highly sensitive (detection to pg or lower levels), and can be used to quantify the relative concentration of the protein of interest. The method depends on incubation of the blot with a substrate that luminesces when exposed to the reporter on the secondary antibody. The light is detected using either photographic film or a charge-coupled device (CCD) camera. The image then is analyzed by densitometry to evaluate the relative amount of protein staining in terms of optical density. By using an appropriate set of molecular weight standards as markers, analysts can estimate molecular weight.

**FLUORESCENCE DETECTION**

Direct fluorescence can be used to detect proteins on blots. Direct fluorescence is simple, rapid, sensitive, and has a greater linear range than enhanced chemiluminescent detection. The advantage of direct fluorescence is the ability to detect many different fluorescent signals. This analysis avoids the need to reprobe the blot. Compared to enhanced chemiluminescence, fluorescence methods are easier to visualize and quantitate on CCD or laser-scanning imaging systems. Some data-acquisition systems permit extending the time of data acquisition to optimize signal-to-noise levels. Fluorescence-labeled blots that can be re-examined are useful for this purpose.

Enhanced chemifluorescence (ECF) is another common fluorescence method. ECF uses secondary antibodies conjugated to either HRP or AP. The enzyme-conjugated antibodies react with specific substrates that produce fluorescence after enzymatic cleavage. Analysts visualize the resulting signals using UV epi-illumination and capture digital images. An ECF signal has a greater linear range than traditional enhanced chemiluminescence. For example, direct fluorescence has a limit of detection in the pg range, and also has about 2 logs of linear dynamic range.

Quantum dots also are an alternative to detect proteins in immunoblot analysis. Quantum dots are a type of probe that can be conjugated to antibodies simultaneously or sequentially to detect multiply labeled antigens, without the need for blot stripping. Similarly, near-infrared (NIR) fluorophore–linked antibody is a method for antibody detection whereby light produced...
from the excitation of a fluorescent dye is measured in a static state. Light measured in a static state allows more precise and accurate detection than light measured in a dynamic state (e.g., chemiluminescence).

RADIOACTIVE DETECTION

Proteins also can be detected by labeling an antigen with a radioactive isotope (e.g., iodine). On the one hand, this method has the advantage that the radioactivity in a band is easy to quantitate by means of time exposure to film and densitometry, or by directly excising the band from the membrane and counting using a scintillation counter. On the other hand, radioactivity also introduces the disadvantage of safety because analysts must manage radioactive material, and analytical laboratories must have a program to control and monitor waste management and individual exposure.

Immunoblot Quantitation

NON-ELECTROPHORETIC QUANTITATION

The quantitation of a specific protein is achieved when the blot procedure is properly optimized and generates a linear response range over a particular time frame. Immunoblot quantitation includes several elements: adequate antigen and antibody concentrations and purity, antibody specificity, blocking conditions, sufficient washes, and the duration and intensity of the signals. Once the exposures are captured on a film or electronically under optimized conditions, analysts use densitometric methods to quantitate results by comparing a specific protein on the blot and on the standard. Analysts can correct results for background by including a negative control.

The intensity of the bands depends on the amount of protein. Different commercial software packages are available for image analysis of bands on a film. Alternatively, digital imaging systems containing CCD cameras usually include software designed to perform data analysis.

ELECTROPHORETIC QUANTITATION

Proteins of various molecular weights are identified by the extrapolation of plots of relative mobilities of prestained proteins of known molecular weight and can be compared to the positive control. Positive controls are trended to determine the limit range of the densitometry results compared to the nominal concentration results. Independent of the detection method, the following criteria must be met for a valid Western blot result.

- Ensure adequate development by minimizing membrane overexposure and visualizing staining controls.
- The prestained molecular weight markers must be visible and must cover the anticipated range.
- The band(s) should have the appropriate location and intensity for the standard, the control, and the protein of interest.
- There should be no blot or staining artifacts that obscure the visualization and interpretation of bands.

METHOD VALIDATION

As outlined by ICH Guideline Q2(R1) Validation of Analytical Procedures: Text and Methodology (effective November 2005) and USP general chapter Validation of Compendial Procedures (1225), a qualitative assay such as the slot/dot blot requires validation of specificity. Specificity is the ability to detect the analyte in the presence of other components. For validation, it should be shown that the particular steps of the slot/dot blot method can detect the antigen when present and do not report false positive results when the antigen is absent. In addition, demonstration of the specificity of the antigen-specific antibodies is part of the specificity evaluation.

USP general chapter (1225) provides guidelines for the validation of analytical procedures, and analysts should consider this resource when they validate immunoblot methods. All methods require a demonstration of the specificity of the antibody to the antigen and the lack of recognition of other proteins and reagents in the matrix. Identity tests require only specificity. Limit tests require specificity and LOD. A sensitivity control incorporated into each test can show that the LOQ is met on each determination to account for potential changes in the sensitivity of the method. A quantitative test requires all ICH validation parameters, including robustness testing.

Demonstration of electrophoretic immunoblot specificity should include the following: stained gels to show protein separation, stained blots to show adequate protein transfer to the membrane, blots with control samples to show the specificity of the conjugate to the primary antibody, and blots that show the binding of the antibody to the appropriate antigen. Method validation also can identify the need for control membranes for each assay, as well as protein sensitivity controls as measures of system suitability.

〈1105〉 IMMUNOLOGICAL TEST METHODS—SURFACE PLASMON RESONANCE

Introduction

Surface plasmon resonance (SPR) optical detection is a useful method for the label-free assays (procedures) that study biomolecular interactions. Commercially available SPR biosensors that incorporate these assays can collect real-time, information-rich data from binding events. These data can be used widely from basic research to drug discovery and development to manufacturing and quality control (QC). SPR can characterize binding events with samples ranging from
proteins, nucleic acids, and small molecules to complex mixtures, lipid vesicles, viruses, bacteria, and eukaryotic cells. Typical quality and safety attributes addressed with SPR analysis include:

- Interaction specificity
- Interaction affinity
- Kinetic binding parameters
- Thermodynamic parameters
- Biologically active concentration of an analyte

This chapter provides an overview of the physics underlying SPR and common instrument configurations, as well as the range of molecules that can be studied and general considerations for experimental design as determined by the assay objective.

**Overview**

**History**

The physical principles of SPR were first explained in the early 1900s, starting with a description of the uneven distribution of light in a diffraction grating spectrum caused by the excitation of surface plasmon waves. A landmark series of experiments showed the optical excitation of surface plasmons under conditions of total internal reflection and fostered detailed studies of the application of SPR for chemical and biological sensing. Since then, SPR’s potential for characterizing thin films and monitoring interactions at metal interfaces has been recognized, and significant research and development have yielded instruments that can quantitatively evaluate the binding interactions of small and large molecules.

**Physics**

SPR is an optical phenomenon that occurs when a thin conducting film is placed between two media that have different refractive indices. In many commercially available instruments, the two media are glass and the sample solution, and the conducting film is preferentially a gold layer applied to the glass, although other conducting metals such as silver have been used. The glass—metal component comprises a solid support that is often referred to as a sensor.

Light applied to the glass under conditions of total internal reflection produces an electromagnetic component that is called an evanescent wave. The evanescent wave penetrates the medium of lower refractive index (typically the sample solution) without losing net energy. The amplitude of the evanescent wave decays exponentially with distance from the surface, roughly one-half of the wavelength of the incident light (e.g., for a light source of 760 nm the evanescent wave penetrates approximately 300 nm).

For a specific combination of wavelength and angle of incident light, electron charge density waves called plasmons are excited in the gold film. As energy is absorbed via the evanescent wave, a decrease in the intensity of the reflected light at a specific angle (the SPR angle) is observed. Analysts can conduct an SPR experiment by fixing the wavelength and varying the angle of incident light.

An increase in mass at the sensor surface caused by a binding interaction between two or more molecules causes a change in the local refractive index (RI) that gives rise to an SPR response, which is observed as a shift in the SPR angle. By monitoring the shift in the SPR angle as a function of time, an analyst can generate a sensorgram (Figure 1). The change in RI is very similar for different proteins, so the SPR measurement depends primarily on the mass change at the sensor surface and is relatively independent of the nature of the molecules being measured.

![Figure 1. Representative sensorgram.](image)

**Instruments**

The main components of commercially available SPR instruments are (1) a light source, typically a high-efficiency light-emitting diode, (2) an optical detector such as a diode-array or charge-coupled device camera, (3) a solid support containing the conducting film and some means for attaching molecules, (4) a sample delivery system, frequently a microfluidic device capable of delivering samples using single serial or parallel injections via single or multiple needles, and (5) a computer with appropriate software for instrument control, data collection, and analysis.

Prism-based and diffraction-grating instrument systems are commercially available. Most prism-based systems follow the Kretschmann configuration (Figure 2). The light is focused onto the sensor surface (away from the samples) via a prism with a refractive index matching that of the surface. In this configuration the incident light does not penetrate the sample solution, which permits SPR measurements for heterogeneous, turbid, or opaque samples. In systems that utilize a diffraction grating (Figure 3) the analyte solution is placed over a plastic surface on which a metal has been deposited. The plastic acts as an attenuated total internal reflection prism in which light reflected from the grating is reflected many times back to the grating surface. In this configuration light passes through the analyte sample solution, and thus turbid or opaque samples are not suitable for measurement. The diffraction grating does permit sampling of a larger surface area and is applicable for SPR measurements of arrays.
The instruments are compatible with a wide range of biological samples and buffers as well as some organic solvents.

**Biomolecular Interactions That Can Be Studied By Assays Using SPR**

A diverse range of biological entities can be studied using SPR, including small molecules (<100 Da), proteins, nucleic acids, lipids, bacteria, viruses, and whole cells. Most published SPR research involves protein–protein interactions, of which antibody–antigen interactions represent a dominant subset. Improvements in instrument sensitivity and experimental protocols have helped analysts make studies of small molecules, lipids, and nucleic acids. Protein interactions with larger entities such as whole cells and some bacteria and viruses are limited by the exponential decay of the evanescent wave as described above. In practice these large molecules can be studied effectively, but the information obtained may be limited to qualitative or semiquantitative (e.g., relative ranking) data.

**Assay Types**

Several types of SPR assays are useful, including binding specificity, concentration analysis, kinetics and affinity analysis, and thermodynamics. Each assay type generates unique information that is helpful for profiling biomolecules.

SPR is also suitable for use in qualitative studies to confirm the specificity of interactions. Analysts can monitor a number of sequential binding events because each individual event yields a mass increase on the sensor chip surface, and all stages in the binding process are monitored. Examples include epitope mapping, antibody isotyping, and immunogenicity measurements.

Most chemical and spectroscopic methods used to quantify proteins (1) measure total protein content, (2) do not distinguish active from inactive molecules, and (3) cannot be used in conjunction with unpurified samples. Because SPR is a noninvasive method (no light penetrates the sample), it can measure small amounts of analyte molecules from complex matrices such as food products, serum or plasma, and cell extracts. Direct or indirect (inhibition or competitive) formats for measuring concentration are possible. SPR biosensors are uniquely suited for measurement of kinetic association and dissociation rate constants from real-time measurement of binding interactions. Affinity can be derived either from interactions that have reached equilibrium or from the ratio of the dissociation and association rate constants. The typical working range for affinity measurements is pM to high µM concentrations. Association rate constants that can be measured typically range from $10^3$ to $10^7$ M$^{-1}$s$^{-1}$ and dissociation rate constants from $10^{-5}$ to $0.5$ s$^{-1}$. By studying temperature dependence of rate and affinity constants, analysts can determine thermodynamic parameters for a binding interaction. Not only can the equilibrium values for changes in enthalpy ($\Delta H$) and entropy ($\Delta S$) associated with complex formation be determined, but transition state energetics can also be evaluated. Subsequent sections of this chapter address the specific details for these different assay types.

**The SPR Assay**

The typical SPR assay involves five steps:

1. Sample and buffer preparation
2. Surface preparation
3. Analyte binding
4. Surface regeneration
5. Data analysis and interpretation

Careful attention to experimental design leads to high-quality data and results. In SPR experiments, mass transport is essential for binding interactions to take place in instruments that use thin-layer flow-cell systems. Analyte molecules
are transferred from the bulk solution to the binding surface via mass transport. When a limitation for binding occurs as a result of fast binding kinetics combined with high surface density, the binding interaction is considered mass-transport limited. In this case, the binding kinetics and complex formation are influenced by the availability of analyte molecules. The advantages and disadvantages of mass-transport–limited binding are discussed later in the application examples.

**Sample and Buffer Preparation**
Both purified and crude samples can be analyzed in a variety of matrices including serum, plasma, cell supernatants, and lysates. Crude samples containing particulates (e.g., cell debris or precipitates) may require clarification in order to help minimize unwanted binding. A short spin (30–60 s) in a benchtop centrifuge or filtration (0.22–1.0 µm) using low–protein-binding filters is recommended. The concentration range for evaluation depends on the experimental objective (yes/no binding, concentration, or kinetic/affinity analysis) as well as the binding affinity of the interacting molecules. In general, sample concentrations an order of magnitude below the equilibrium dissociation constant ($K_D$) can be detected by SPR, but determination of an exact concentration is influenced by the analyte size (large vs. small molecules), binding specificity, and overall biological activity of the samples. Most biological buffers and several organic solvents can be used in SPR experiments. The addition of salts and detergents to buffer solutions frequently can stabilize biomolecules. High-quality grade (e.g., molecular biology grade or higher) buffer components should be used. To simplify experiments, analysts should add only components that are absolutely required for biological activity or function. Buffers should be filtered and degassed before use.

**Surface Preparation**
Surface preparation involves the attachment of one of the binding partners to a solid support (surface). This process is frequently referred to as immobilization, and the resulting surface with the attached biomolecule is the sensor for the experiment. The choices of binding partner, solid support, and immobilization method are influenced by (1) the nature and demands of the application or experimental objective; (2) the availability of surfaces with different properties (e.g., charge density, hydrophobicity, or hydrophilicity); (3) the characteristics and supply of biomolecule to be used for immobilization; and, most importantly, that (4) biological activity be maintained and binding sites be available to interacting partners. Depending on the experimental objective, homogeneous or orientation-specific attachment of biomolecules also may be desired. The two main categories of immobilization methods are (1) direct immobilization, in which the molecule is covalently attached to the surface, and (2) indirect or capture immobilization, which takes advantage of tags or native groups on the protein or biomolecules (Table 1).

**Table 1. Surface Preparation Techniques**

<table>
<thead>
<tr>
<th>Chemistry</th>
<th>Immobilization Method</th>
<th>Biomolecules</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amine</td>
<td>Direct</td>
<td>Proteins, peptides</td>
<td>Amino terminus, Lys residues</td>
</tr>
<tr>
<td>Thiol—native</td>
<td>Direct</td>
<td>Proteins, peptides</td>
<td>Native Cys residue</td>
</tr>
<tr>
<td>Thiol—added</td>
<td>Direct</td>
<td>Proteins, peptides</td>
<td>Carboxyl groups derivatized</td>
</tr>
<tr>
<td>Aldehyde</td>
<td>Direct</td>
<td>Glycoproteins</td>
<td>Gis–diol required</td>
</tr>
<tr>
<td>Biotin capture</td>
<td>Indirect</td>
<td>Biotinylated peptides, nucleic acids, proteins</td>
<td>Stable, irreversible capture</td>
</tr>
<tr>
<td>Affinity tags</td>
<td>Indirect</td>
<td>Proteins, peptides</td>
<td>His, Glutathione S-transferase (GST), etc.</td>
</tr>
<tr>
<td>Protein A, Protein G</td>
<td>Indirect</td>
<td>Antibodies, IgG-tagged molecules</td>
<td>IgG species–dependent</td>
</tr>
<tr>
<td>Protein A, Protein G</td>
<td>Indirect</td>
<td>Biomolecules specific to the capturing antibody</td>
<td>Mono- or polyclonal antibodies may be suitable—testing recommended</td>
</tr>
<tr>
<td>Hydrophobic adsorption, membrane capture</td>
<td>Indirect</td>
<td>Lipids, membranes, membrane-associated proteins</td>
<td>Monolayer or bilayer attachment possible</td>
</tr>
</tbody>
</table>

**Direct Immobilization:** For direct immobilization, several chemistries are available for attaching proteins or other biomolecules to the surface. The properties of the surface determine the specific sequence of steps and length of time required to prepare the surface. Many commercially available surfaces have a biologically compatible layer (e.g., a hydrogel) that contains functional groups such as carboxyl that can be used for immobilization. To ensure binding specificity, the purity of the biomolecule that is attached to the surface should be 95% or greater and the required concentration should range from 1 to 1000 µg/mL. Direct immobilization chemistries frequently result in heterogeneous surfaces because of random orientation of biomolecules on the surface. Immobilization via free primary amine groups such as lysine residues in proteins or the amino terminus of proteins or peptides is one of the most generally applicable covalent chemistries for attaching proteins to a surface. Carboxyl groups on the surface are converted to reactive esters using a mixture of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) or sulfo-NHS (sNHS). The protein or biomolecule is applied in high concentrations (mg/mL) to maximize the efficiency of amine coupling. Finally, free esters are blocked with ethanolamine. The contact time with the surface, the protein concentration, or the EDC/NHS concentration can be varied to adjust the immobilization level.

When amine coupling interferes with the binding site, the biomolecule can be attached using alternative coupling chemistries or a high-affinity capture approach. For example, for biomolecules with free thiol groups (typically cysteine residues), a disulfide group is introduced by treating the surface with NHS and EDC to attach 2-(2-pyridinylthio)ethaneamine (PDEA). Adding the biomolecule to the surface results in thiol–disulfide exchange, and excess PDEA groups are inactivated with cysteine–HCl. If the biomolecule lacks a free thiol group, a reactive disulfide (PDEA) can be linked to carboxyl groups. Subsequently the pyridyl disulfide groups can be
attached to thiol groups on the surface that have been derivatized via injection of NHS and EDC, followed by
cystamine, then reduction with dithioerythritol (DTE) or dithiothreitol (DTT). Attachment of maleimide groups to
the surface makes possible an alternative form of immobilization via thiol groups in which a stable thioether bond
is formed. Surfaces prepared using this method have the capacity to withstand basic pH (> 9.5) and reducing
agents such as β-mercaptopethanol and dithiothreitol. Several heterobifunctional reagents are available
commercially for introduction of reactive maleimido groups to the surface, including sulfo-MBS
\( (m\text{-maleimidobenzoyl-N-hydroxysulfosuccinimide ester}) \), sulfo-SMCC
\( (\text{sulfosuccinimidyl-4-(N-maleimidomethyl)cylohexane-1-carboxylate}) \), GMBS
\( [N-(\gamma\text{-maleimidobutyryloxy)sulfosuccinimide ester}] \), EMCH
\( [N-(\text{maleimidocaproic acid})\text{-hydrazide}] \) or BMPH
\( [N-(\text{maleimidopropionic acid})\text{-hydrazide}] \). For biomolecules
containing either native aldehyde groups or dis–diols, which may be converted into aldehydes by mild oxidation,
surface attachment via a hydrazide bond is an option. Hydrazide groups on the sensor surface react with aldehyde
groups on the biomolecule to form a stable bond. Immobilization via aldehyde groups is most useful for
glycoconjugates, glycoproteins, and polysaccharides.

**Indirect (High-affinity Capture) Immobilization:** Indirect or high-affinity capture immobilization approaches use tags
commonly used for protein purification. This technique exploits the high-affinity capture of the biomolecule by a
capturing molecule that has been immobilized covalently using one of the techniques described above. The
requirement for biomolecular purity is less stringent for indirect versus direct immobilization because the capturing
step for the biomolecule can also provide purification. Indirect immobilization frequently yields a homogenous
surface because all biomolecules are oriented similarly via the tag. The affinity between the biomolecule and its
capturing agent should be sufficiently high to ensure little or no dissociation from the surface for the duration of an
analysis cycle. Monoclonal antibodies are frequently used as capture molecules. For example anti-GST antibodies
can be attached to the sensor chip surface via amine chemistry in order to capture GST-tagged molecules. Protein
A, Protein G, and anti-IgG antibodies are useful capturing molecules for use with antibodies.

The high-affinity interaction between streptavidin or related molecules and biotin \( (K_a \approx 10^{-15} \text{ M}) \) makes it a useful
system for the capture of biotinylated molecules (e.g., proteins, peptides, nucleic acids, membranes, and
liposomes). Frequently, the biotin-binding protein is attached to the surface using primary amine groups. Because
of the high affinity of the interaction, biotinylated molecules are considered permanently immobilized, and in contrast to most other capture approaches biotinylated molecules cannot be removed without damaging the
surface. Histidine (His)-tagged recombinant proteins can be captured via nickel–NTA chemistry or covalently
immobilized anti-His antibodies.

Lipids and membrane-associated proteins can be captured to the surface as either a lipid monolayer or bilayer. Lipids
from micelles or liposomes adsorb to a hydrophobic surface, creating a lipid monolayer with the hydrophobic
lipid tails oriented toward the solid support and the hydrophilic heads towards the aqueous sample. This approach
provides a stable environment for proteins associated with a membrane surface or partially inserted into the
membrane, but it is not ideal for transmembrane proteins because the resulting surface presents only half the
membrane structure for binding interactions. Intact membrane structures (lipid bilayers) with associated or
incorporated proteins can be captured by preparing liposomes with a specific antigenic component or with
biotinylated lipids, allowing capture of the liposomes with immobilized antibody or streptavidin, respectively.

**Additional Considerations:** Once the biomolecule has been attached to the sensor using either a direct or indirect
immobilization approach, analysts should assess the baseline stability of the newly created surface. If the baseline is
decreasing (downward drift), the most likely cause is the presence of unattached biomolecules, possibly because of
self-association or aggregation. If the baseline is increasing (upward drift) refolding or re-orientation may be causing
the change. In either case, the newly created surface should be conditioned before use by one or more of the
following: (1) multiple injections of biologically compatible buffer; (2) washing the surface with buffer at a fast flow
rate; (3) multiple injections of either high ionic strength (e.g., 1 M NaCl) or detergent (e.g., 20 mM CHAPS or 0.05%
Polyasorbate 20 (P20)) solutions; or (4) repeated analyte binding and regeneration injections. NOTE:
recommendations (3) and (4) should be used only if the activity of the biomolecule in the presence of these reagents
has been evaluated previously.

Large baseline drifts caused by low-affinity capture may be overcome by using EDC/NHS as a cross-linking step, but
this may compromise biomolecule activity if active sites of the biomolecule are involved in the cross-linkages. The
effect of cross-linking on biomolecule activity must be tested empirically for each biomolecule–analyte system. In
general, cross-linking should be as brief as possible: 15 s is often sufficient to achieve acceptable baseline stability
without compromising biomolecule activity.

**How Much to Immobilize:** The amount of biomolecule to immobilize depends on the experimental objective.
Equations 1 and 2 are useful for calculating the appropriate surface density:

\[
R_{\text{max}} = \left(\frac{\text{MW}_{\text{A}}}{\text{MW}_{\text{L}}} \right) \times R_e \times S_m \quad [\text{Equation 1}]
\]

\[
R_e = R_{\text{max}} \times \left(\frac{1}{S_m} \right) \times \left(\frac{\text{MW}_{\text{A}}}{\text{MW}_{\text{L}}} \right) \quad [\text{Equation 2}]
\]

- \( R_{\text{max}} \) = theoretical maximum binding response (assuming a surface that is 100% active and 100% bound with analyte)
- \( R_e \) = response of the immobilized molecule
- \( \text{MW}_{\text{A}} \) = molecular weight of the analyte
- \( \text{MW}_{\text{L}} \) = molecular weight of the immobilized molecule
- \( S_m \) = molar binding stoichiometry

For kinetic experiments, a low density of immobilized molecule is preferred in order to avoid steric hindrance,
aggregation, and/or mass-transport–limited binding. Low density is defined as \( R_e \) that limits \( R_{\text{max}} \) to 5–50 response
units. For other applications, e.g., concentration analysis where mass-transport–limited binding is desired, \( R_{\text{max}} \)
can be 100–200 times higher than for kinetic experiments provided that steric hindrance or aggregation are not induced. Specific recommendations for immobilization density are included in the application examples for this chapter.

**Analyte Binding**

Samples that will be evaluated for binding using SPR do not require the same purity as biomolecules intended for direct immobilization onto the surface. Because the light source does not penetrate the sample, turbid or opaque samples can be analyzed by SPR. Whenever practical, samples should be clarified according to the recommendations given under Sample and Buffer Preparation, and buffer additives should be minimized, including only the amount required for biological activity.

Differences between the refractive index of the bulk and sample buffers give rise to a response. The use of control surfaces and samples aids in demonstrating binding specificity for the molecules in SPR. For direct immobilization methods, suitable control surfaces can be (1) the sensor surface without any modification or biomolecule attached, (2) a surface that has been chemically treated in the same manner as the surface containing the biomolecule, or (3) a related but known nonbinding biomolecule. For surfaces prepared using indirect (capture) immobilization the capturing molecule in the absence of the tagged binding partner should be used as the control surface. The difference in response between the control and active surfaces gives an initial indication of the binding specificity.

Concentration-dependent responses and inhibition of binding by incubating the sample with the biomolecule on the surface can further establish the binding specificity.

If nonspecific or unwanted binding is observed, analysts should determine the source. Frequently changes in pH or ionic strength of the buffers used in the experiment will reduce or eliminate the unwanted binding. Additional suggestions for reducing nonspecific binding are summarized in Table 2.

<table>
<thead>
<tr>
<th>Category</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experimental Design</strong></td>
<td>1. Optimize running buffers:</td>
</tr>
<tr>
<td></td>
<td>A. increase salt (150 to 500 mM)</td>
</tr>
<tr>
<td></td>
<td>B. add detergent (0.001% to 0.05%)</td>
</tr>
<tr>
<td></td>
<td>C. match composition of sample and running buffers</td>
</tr>
<tr>
<td></td>
<td>2. Change ligand immobilization method</td>
</tr>
<tr>
<td></td>
<td>3. Evaluate ligand quality</td>
</tr>
<tr>
<td></td>
<td>4. Increase or decrease temperature in detection chamber</td>
</tr>
<tr>
<td><strong>Choice of Surface</strong></td>
<td>1. Change properties of sensor surface:</td>
</tr>
<tr>
<td></td>
<td>A. reduce electrostatic interactions</td>
</tr>
<tr>
<td></td>
<td>B. evaluate hydrophobic vs. hydrophilic character of surface</td>
</tr>
<tr>
<td></td>
<td>C. consider alternative ligand to use for control surface</td>
</tr>
<tr>
<td></td>
<td>2. Pre-immobilize amino—PEG</td>
</tr>
<tr>
<td></td>
<td>3. Change blocking molecule (e.g., ethylenediamine)</td>
</tr>
<tr>
<td><strong>Additions to Sample</strong></td>
<td>1. Add nonspecific binding reducer to sample:</td>
</tr>
<tr>
<td></td>
<td>A. increase ionic strength of running and sample buffers (e.g. 150 to 500 mM NaCl)</td>
</tr>
<tr>
<td></td>
<td>B. add detergent to running and sample buffers (e.g. 0.001% to 0.05% surfactant P20)</td>
</tr>
<tr>
<td></td>
<td>C. add soluble carboxymethyl dextran (1–10 mg/mL, for dextran-based surfaces only)</td>
</tr>
<tr>
<td></td>
<td>2. Simplify sample buffer—include only components required for biological activity</td>
</tr>
<tr>
<td></td>
<td>3. Evaluate analyte quality</td>
</tr>
</tbody>
</table>

Equations 1 and 2 are also useful for assessing surface activity. The higher the binding response, the more active the surface is unless the observed binding response exceeds the calculated $R_{\text{max}}$ value. In this case, the molar binding stoichiometry is incorrect, the analyte molecule is aggregated, or the analyte is binding nonspecifically to the surface. Binding responses that are low (< 10% of $R_{\text{max}}$) suggest that the analyte concentration selected for the experiment is too low or that the surface activity of the immobilized molecule is low. In the former case, increasing the analyte concentration should increase the binding response, and in the latter situation using a different immobilization method may be helpful.

**Surface Regeneration**

Surface regeneration refers to the process of removing bound analyte from the surface in order to reuse the surface for subsequent binding interactions. In some instances, complex dissociation is fast and bound analyte is simply washed away with buffer, so regeneration is not needed. Alternatively, the instrument configuration may allow multiple samples to be injected either sequentially or in parallel across several immobilized surfaces simultaneously, thereby limiting the need for regeneration. Inadequate surface regeneration may affect the reproducibility of an assay and negatively affect the overall quality of the resulting data. To identify the correct conditions, analysts should consider the nature of the specific interaction and the experimental objective. For example, a slight baseline drift will not affect the results when a simple yes/no answer is sought, but in concentration determination or kinetic studies, optimization of the regeneration step is critical.

Most biochemical interactions involve non-covalent bonds such as hydrogen, electrostatic, van der Waals, and hydrophobic bonds. Because the combination of physical forces responsible for binding and the regeneration conditions critical for not causing irreversible conformational changes are unknown for most interactions, the final conditions must be evaluated empirically.

The ideal condition for regeneration dissociates all the bound material without affecting the biological properties of the immobilized biomolecule. An incomplete regeneration or too stringent conditions may result in decreased analyte binding capacity in subsequent cycles because of either blocking of binding sites by nondissociated analyte.
or partial denaturation of the biomolecule. Regeneration buffers and solutions can be divided into different classes by the effect they have on the interaction. Any combination of buffers can be used. The major classes of regeneration buffers are: acidic, basic, ionic/chaotropic, detergent, hydrophobic/nonpolar, and chelating (see Table 3 for examples of each class). Analysts should start with mild conditions, moving progressively to more harsh conditions. In many cases, especially when one is working with antibodies, change in pH is the most effective method of regenerating the surface. When analysts use pH change, the contact times should be short, one-half to 2 min. When analysts use high ionic strength or chaotropes, longer contact times of 2–4 min are usually effective.

Table 3. Examples of Regeneration Solutions

<table>
<thead>
<tr>
<th>Acid</th>
<th>Base</th>
<th>Ionic/Chaotropic</th>
<th>Detergent</th>
<th>Hydrophobic/Nonpolar</th>
<th>Chelating</th>
</tr>
</thead>
<tbody>
<tr>
<td>1–100 mM HCl</td>
<td>1–100 mM NaOH</td>
<td>0.5–5 M NaCl</td>
<td>0.02%–0.5% SDS</td>
<td>25%–100% ethylene glycol</td>
<td>10–20 mM EDTA or EGTA</td>
</tr>
<tr>
<td>10–100 mM glycine, pH 1.3–3.0</td>
<td>10–100 mM glycine, pH 9.0–10.0</td>
<td>1–4 M MgCl₂</td>
<td>40 mM octylene glycol + 20 mM CHAPS</td>
<td>5%–50% DMSO</td>
<td>10–200 mM imidazole</td>
</tr>
<tr>
<td>10–100 mM phosphoric acid</td>
<td>1 M ethanolaime HCl, pH 9.0 or above</td>
<td>1 M KSCN</td>
<td>40 mMoctylglucoside, 40 mM octylglucoside</td>
<td>1%–10% acetonitrile</td>
<td></td>
</tr>
<tr>
<td>0.1% TFA</td>
<td>100 mM sodium carbonate + 1 M NaCl, pH 9–11</td>
<td>2–6 M guanidine HCl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 mM Formic acid</td>
<td>20–100 mM NaOH containing 0.5% surfactant P20 or 0.05% SDS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The purpose of optimizing the regeneration conditions is to find the mildest possible regeneration solution that completely dissociates the complex. Analysts should maintain a constant level of activity over the binding–regeneration cycles even if the baseline changes a little. Repeated cycles of analyte binding followed by regeneration of the surface will provide insight into the overall performance of the surface. Ideally the surface performance should be evaluated for the same number of cycles that will be used during the SPR experiment.

The surface must be monitored for signs of accumulation and also degradation of the immobilized ligand (Figure 4). This can be accomplished by monitoring both the baseline at the beginning of each injection cycle and binding signal (slope or bound response) of a quality control sample. Appropriately defined acceptance criteria for system suitability such as baseline drift and quality control performance help to monitor the integrity of the immobilized ligand on the surface.

Figure 4. Evaluating surface performance (A) accumulation on surface and (B) degradation of immobilized ligand.

If the binding response is slowly decreasing, there are two possible explanations:

1. If the baseline of the raw data sensorgram remains constant but the binding response still decreases, the regeneration conditions cause an irreversible change to the biomolecule that decreases the binding capacity of the surface, which in turn decreases the amount of analyte that can be bound on the surface. Analysts can decrease the strength of the regeneration solution slightly or can change to another regeneration solution of equal strength within the same class.
Data Analysis and Interpretation

Analysis and interpretation of the data are specific to the experimental objective. Several data analysis programs exist to aid in the calculation of kinetics and affinity constants from SPR data. The validity and quality of the results are linked directly to experimental design. The fitting process is purely mathematical, without regard to the biological significance of the values obtained.

Data Analysis Algorithm: Global analysis seeks a single set of kinetic rate constants for all of the analyte concentrations used in the experiment. Using a data-fitting algorithm such as Marquardt-Levenberg the data analysis software begins an iterative process starting with an initial approximation to find the best set of parameters that produces agreement between the experimental data (sensorgram) and the calculated fit to the data. The iterative process continues until the difference between the experimental and calculated (theoretical) curves is minimized as measured by the sum of the squared residuals.

Preparing the Data for Analysis: Before conducting kinetic analysis, analysts should inspect the experimental data visually for anomalies or artifacts such as baseline disturbances or out-of-range data (often due to air bubbles) lasting for a predefined time period (e.g., 4–8 s). Outliers should be removed from the data set according to pre-established criteria. Nonessential data, such as capture or regeneration injections, should be removed from the sensorgram, and the data at each analyte concentration should be adjusted using the double-referencing procedure described below. Before analysis the raw data should be processed in the following manner:

- Align the injection start to zero seconds for all concentrations and buffer injections for both the reference and active surfaces.
- Align the baseline to zero response for all sensorgrams.
- Subtract the reference surface sensorgram from the active surface sensorgram in order to create a corrected data set.
- Subtract the corrected buffer sensorgram from the sensorgrams at different concentrations in order to create a double-referenced data set.

The double-referencing procedure removes systematic errors (e.g., instrument noise) and low levels (less than 5% of total binding response) of nonspecific binding. It should not be used to correct for significant nonspecific binding events because this can lead to erroneous measurements.

When analyzing the data for kinetic information, analysts use the association (injection) and dissociation (buffer flow) phases for all of the concentrations in the series. For steady-state affinity analysis, the response at equilibrium $R_{eq}$ (data plateau or no change in response vs. time) is measured for each sensorgram to create a binding isotherm with $R_{eq}$ vs concentration. This isotherm is analyzed using the equations described below.

Kinetics and Steady-state Affinity Models: The Langmuir kinetic model assumes a 1:1 interaction between the binding partners so that

$$A + B \xrightarrow{k_a} AB$$

The association and dissociation rate constants are defined below:

$$\frac{d[AB]}{dt} = k_a \times [A] \times [B]$$

$$-\frac{d[AB]}{dt} = k_d \times [AB]$$

Combining these two equations and defining $[B_{tot}] = [B_{tot} - AB]$, the net rate expression is

$$\frac{d[AB]}{dt} = k_a \times [A_{tot}] \times [B_{tot} - AB] - k_d \times [AB]$$

which can be translated into terms from the SPR experiment as follows:

$$\frac{dR}{dt} = k_a \times C \times (R_{max} - R) - k_d \times R$$
where \( R \) is the binding response at any point along the sensogram and \( C \) is the known analyte concentration. Using global analysis as described above, \( k_a \), \( k_d \), and \( R_{\text{max}} \) are calculated from the experimental data using the rate equations shown below:

**Association:** \( \frac{\text{d}R}{\text{d}t} = k_a \times C \times (R_{\text{max}} - R) - k_d \times R \)

**Dissociation:** \( \frac{\text{d}R}{\text{d}t} = -k_d \times R \)

Because the concentration of analyte is zero during dissociation, the rate equation for dissociation depends only on the response, \( R \), and the dissociation rate constant, \( k_d \).

Application of the equilibrium condition where the complex formation (association) equals complex decay (dissociation)

\[ k_a \times [A] \times [B] = k_d \times [AB] \]

yields the following equation for the equilibrium dissociation constant

\[ K_D = \frac{[A] \times [B]}{[AB]} \frac{C(R_{\text{max}} - R_{eq})}{R_{eq}} \]

where \( R_{eq} \) is the binding response at equilibrium that is measured in the experiment for a given analyte concentration and \( C \). \( K_D \) and \( R_{max} \) are calculated using global analysis.

Kinetics binding models can be used to describe non-1:1 interactions, e.g., bivalent interactions that occur if an antibody is used as the analyte, heterogeneity in binding partners, conformational change, or more complex interactions such as cooperative binding. SPR analysts are cautioned against using more complex models to assess data unless experimental design has been confirmed.

**Assessing the Fit:** The quality and validity of the fit to the kinetic data can be assessed by (1) visual inspection of the agreement between the experimental and calculated curves, (2) the size and the shape of residual plots, (3) the biological relevance of the results, and (4) statistical parameters such as \( \chi^2 \) (average of squared residuals), and standard error (SE), \( T \)-value or \( U \) (uniqueness) factor. The best parameter fit to the experimental data should be superimposed on the curve for each concentration in the experiment. The residual plot visualizes the difference between the calculated and experimental data. The shape of the residuals should be random without trending (waviness or curving up or down). The height of the residual plot should reflect the instrument noise. Further, \( \chi^2 \) should be minimized for a good fit with values that depend on the instrument noise, number of data points, and overall binding response. The parameter values should be considered for biological and experimental relevance. For example, is the calculated \( k_a \) value slow when the interaction is known to be fast, or is the calculated \( R_{max} \) value higher than the value that was calculated using Equations 1 and 2. Parameter significance is evaluated based on standard error, \( T \)-values, and \( U \) factor. Parameters that are significant cannot be changed without affecting the quality of the fit. All of the criteria should be within acceptable limits.

A similar set of criteria can be used for assessing the fit to steady-state affinity data, but because there are fewer data points (6–12 total, depending on the number of concentrations used), the statistical parameters and residual plots are less predictive of fit quality. Visual inspection of the agreement between the experimental and calculated binding isotherms and the parameter relevance are good tools to use for assessing the fit. Additionally, according to the relationship between concentration, \( K_D \), and \( R_{max} \) when concentration equals the \( K_D \) for the interaction \( R \) equals 50% of \( R_{max} \). Confirming that the analyzed data follow this relationship provides another way to check the validity of the calculated result. When it is practical to calculate the \( K_D \) using both kinetic and steady-state analysis approaches, the \( K_D \) values should agree within experimental error.

**Addressing a Lack of Fit:** When the data do not fit or the parameter values do not make sense, often the problem can be resolved by a systematic approach that considers potential sources for deviations and tests each hypothesis. Items to consider include reagent purity, immobilization chemistry or surface density, analyte concentration errors, nonspecific binding, loss of ligand activity, or mass-transport–limited binding. Reviewing the raw (uncorrected) data helps determine the source of nonspecific binding or concentration errors. Table 4 lists potential sources for deviations from 1:1 binding and recommended actions.

### Table 4. Common Sources for Deviation from 1:1 Binding Kinetics

<table>
<thead>
<tr>
<th>Source of Deviation</th>
<th>Recommended Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonzero baseline before injection</td>
<td>Normalize response to zero and reanalyze</td>
</tr>
<tr>
<td>Incorrect injection start and stop times or poorly defined injection start/stop</td>
<td>Adjust injection start/stop</td>
</tr>
<tr>
<td></td>
<td>Remove sensogram artifacts (e.g., injection or air bubble spikes)</td>
</tr>
<tr>
<td>Concentration input errors</td>
<td>Verify concentration values and reanalyze</td>
</tr>
<tr>
<td>Bulk refractive index contribution too high</td>
<td>Use double-referencing approach before analysis</td>
</tr>
<tr>
<td></td>
<td>Set RI = 0</td>
</tr>
<tr>
<td>Mass-transport–limited binding</td>
<td>Vary flow rate (slow to fast) for a single concentration and overlay sensograms (should be identical for same association and dissociation time)</td>
</tr>
<tr>
<td></td>
<td>Include mass transport term, ( k_n ), in fitting model</td>
</tr>
<tr>
<td></td>
<td>Reduce surface density</td>
</tr>
</tbody>
</table>

Not official text. Please refer to the currently official version of the applicable USP–NF or FCC standard for compliance purposes.
Table 4. Common Sources for Deviation from 1:1 Binding Kinetics (continued)

<table>
<thead>
<tr>
<th>Source of Deviation</th>
<th>Recommended Action</th>
</tr>
</thead>
</table>
| Nonspecific binding  | • Change immobilization chemistry  
                        • Change sensor surface properties  
                        • Buffer additives—add or minimize  
                        • Reagent purity—repurify samples |
| Loss in binding partner activity | • Change immobilization chemistry  
                                 • Change regeneration solution  
                                 • Re-analyze data using local instead of global parameter fitting for \( R_{\text{max}} \) |
| Multi-valent binding interaction | • Immobilize multivalent binding partner |

Recommendations for data analysis will be introduced in the subsequent sections of this chapter.

**Application 1—Immunogenicity Assessment:** SPR has emerged as a powerful technique for assessing immunogenicity of protein therapeutics. An advantage of this platform for detecting antibodies in serum (or plasma) samples is that it allows label-free detection based on mass accumulation in real time, which potentially allows detection of low-affinity antibodies of all classes and subclasses. This technology is useful both for screening assays (first-tier immunoassays that are used to detect the presence of antibodies capable of binding to a protein therapeutic) and for confirmation assays. Characterization assays are used to detect all generated antibodies that bind to the protein and can include analysis of antibody concentration, isotype(s) represented, relative binding affinity, and binding specificity. A limitation is that SPR it is not appropriate for determination of the neutralizing capability of antibodies, which is best determined using cell-based biological assays. When designing and validating SPR assays for immunogenicity assessment, analysts should consider critical parameters including protein immobilization to ensure immunological reactivity, immobilized protein stability, and surface regeneration conditions.

**Protein Immobilization:** The first step in the development of immunogenicity assessment assays is to identify the optimum mechanism for immobilization of the target protein. When considering the target density for immobilization, analysts often recommend that a high-density surface be used. The advantage of a high-density surface is that it maximizes the opportunity that anti-therapeutic antibodies will come in contact with an immobilized ligand. A high-density surface also provides excellent assay sensitivity. An important aspect of these assays is that the chemistry chosen for immobilization should provide random orientation rather than a site-directed orientation so that all potential epitopes on the therapeutic protein are available for binding by the anti-therapeutic antibodies. The effectiveness of immobilization is determined by evaluating the ability of positive control antibodies to bind to the immobilized protein. When evaluating the effectiveness of immobilization, analysts should test multiple antibodies with different epitope specificities. When panels of antibodies that cover a range of affinities and bind to different epitopes on the target protein are all capable of binding, this provides confidence that antibodies contained in clinical specimens also will be detected. If any of the positive control antibodies do not demonstrate binding, this suggests that the immobilization is not optimal and should be modified. Although SPR is demonstrably efficient at detecting low-affinity antibodies, analysts should confirm that the immobilization protocol chosen is effective for detection of low- and high-affinity antibodies.

**Protein Stability Upon Immobilization:** The positive control antibody must be able to bind to the immobilized protein in order for an assay result to be acceptable. This confirmation of binding provides confidence that if antibodies against a protein are present in a sample, they will bind to the immobilized protein on the surface of the sensor. Because SPR relies on re-using the immobilized protein surface for multiple analyses, a regeneration protocol is required to effectively remove any bound material from the immobilized protein. This regeneration procedure is based on the ability to remove bound material without damaging or removing the immobilized protein. Several regeneration protocols can be used, and most often the regeneration solution is an acidic solution, commonly dilute HCl. The immobilized protein must remain intact and functional after repeated regeneration steps.

Because the immobilized protein will be used routinely for multiple analyses involving repeated cycles of serum samples, analysts should verify the stability of the immobilized protein after regeneration cycles. The stability of the immobilized protein can be monitored effectively by tracking the response units after regeneration and also after addition of positive control antibody. If there is a change in baseline or a decrease in the magnitude of binding by the positive control antibody, then the immobilized protein likely is no longer suitable for further analyses. The stability following regeneration should be established during assay development and should be confirmed during assay validation. In order to monitor the performance of the sensor during an assay, analysts should periodically test a positive control sample during an assay run. If the performance of the positive control samples indicates the immobilized protein has been compromised, analysts should re-analyze test samples obtained after the performance of the assay dropped below acceptable limits. Acceptance parameters for immobilization may vary by compound and should be established for each assay.

**Availability of Epitopes After Immobilization:** Once the protein is immobilized, the availability of multiple epitopes should be confirmed. Ideally this is done by testing for binding of positive control antibodies with different epitope specificity. One method for testing epitope availability is to use a panel of monoclonal antibodies that are known to recognize different regions of the protein. If the protein has been randomly immobilized, all the different positive control antibodies should be able to bind. The reason for evaluating epitope availability is to prevent false-negative results when serum samples are evaluated. If the immobilization is not random, it would be possible to consistently immobilize the protein via a specific epitope, thus making that epitope unavailable for binding by an antibody. Another possibility is that chemical modification of the protein to facilitate immobilization altered the protein’s conformation.
Surface Regeneration and Subsequent Protein Stability: Using the previous guidelines, analysts should monitor the surface for signs of accumulation and degradation of the immobilized ligand and discontinue use when necessary. For example, when the binding capacity of a positive control antibody (diluted in test serum) drops below 80% of initial capacity the surface should not be used.

Assay Cut-point Determination: When performing assays to determine if a serum sample contains antibodies against a protein, analysts sometimes observe a background level of binding. That background binding can vary depending on the nature of the immobilized protein and also the patient population being tested. In order to determine if a test sample contains antibodies, analysts compare binding to control samples that do not contain antibodies against the protein. A cut-point is established, and when a sample contains antibodies the binding is greater than that cut-point. Analysts determine the assay cut-point by analyzing a series of serum samples that do not contain antibodies against the immobilized protein and then performing statistical analysis to determine the level of binding consistent with a sample that does not contain antibodies. The cut-point should be established using the same conditions that will be used for sample analysis. Although different approaches are used for determining a cut-point, a common approach is to establish the mean from the binding of 50–100 serum samples from healthy volunteers and set the cut-point at 95% (equivalent to the mean plus 1.645 times the standard deviation for a normal distribution). Analysts should remove statistical outliers from the calculations because their inclusion can cause a high bias and raise the cut-point. This higher cut-point will result in identification of fewer samples with antibodies against the immobilized protein. The statistically evaluated cut-point is the response unit value that serum samples must exceed to be considered positive for the presence of antibodies against the therapeutic protein. An important feature of cut-point determination is that it may be different in different patient populations. For example, patients with inflammatory disease, may show a higher level of nonspecific reactivity compared to a normal population. This higher level of nonspecific binding would result in samples being identified as positive when they did not contain any antibodies specific for the protein. When this situation arises, predose serum samples can be used to establish a new patient population–specific mean and assay cut-point.

Analytical Procedure Development and Validation: Once the stability of the immobilized protein is confirmed, a regeneration procedure has been defined, and the cut-point established, the antibody testing method can be developed and validated. The conditions used for analyzing samples should be identical to those used to establish the assay cut-point. An important parameter to consider is the optimal dilution of the serum sample. Increasing the dilution factor reduces nonspecific binding by serum proteins but also reduces overall sensitivity. Most antibody assessment procedures use between 5% and 50% serum. As the percentage of serum that is tested decreases, the percentage of the binding signal that is due to nonspecific interaction also decreases, and subsequently the percentage of the signal mediated by antibodies binding to the immobilized protein increases. Besides dilution, other means to reduce nonspecific interaction include adding surfactants, increasing salt concentration, adding BSA or HSA, or adding soluble sensor surface support material such as carboxymethyl dextran or alginate to the dilution and running buffer.

Other important variables to optimize include flow rate and sample volume. The combination of flow rate and sample volume defines the contact time, the length of time during which a given sample is in contact with the immobilized protein. The longer a sample is in contact with the immobilized protein, the greater the chances for antibody binding. The next important aspect to consider is verification that initial binding is a result of an antibody and not some other serum component. This can be accomplished by adding an anti-human immunoglobulin reagent and monitoring subsequent binding. If the initial binding observed was due to an anti-protein antibody, this reagent will bind to that antibody (the anti-protein antibody remains bound to the immobilized protein). When a therapeutic monoclonal antibody is the immobilized protein, the confirmatory reagent must be screened and verified not to bind directly to the immobilized therapeutic monoclonal antibody. One option here is the immobilization of the Fab’ fragment rather than the intact therapeutic monoclonal antibody. The confirmatory reagent must be verified for specificity. Once all of the parameters are optimized, the assay can be validated. Validation parameters include those typically associated with immunoassays (precision, specificity, sensitivity, and robustness) as well as parameters specific to SPR assays (protein immobilization, stability of immobilized surface, and number of regeneration cycles).

Interference by Serum Components: Depending on the immobilized protein, serum components other than antibodies specifically directed against the immobilized protein possibly could bind to the immobilized surface. It is also possible that serum components that block the ability of antibodies to bind to the immobilized protein could be present. Both of these can be evaluated by testing the binding of serum samples from the target subject population that are known not to contain antibodies against the immobilized protein and then monitoring to determine if any binding does occur. If nonspecific binding is identified, steps can be taken to reduce or eliminate it. These steps can include pretreatment of samples to remove the nonspecific reactant, addition of surfactant, or alteration of salt concentration in sample buffers to reduce nonspecific binding.

Analysts should verify that serum samples do not contain agents that are capable of inhibiting antibody binding to the immobilized protein (these could include soluble forms of the immobilized protein or soluble receptors that could bind to the immobilized protein and block binding of the antibodies to the immobilized protein). Analysts can add the positive control antibody to target serum samples and can evaluate binding. If binding is inhibited by the target serum samples compared with binding to normal human serum samples, steps can be taken to remove the inhibiting agent. Failure to identify target serum interference can result in either false-positive or false-negative results.

Implementation of Multiplex Assays: When a therapeutic protein is a second-generation product that has been modified from an original therapeutic protein (e.g., via pegylation or increased glycosylation), the presence of antibodies against both the original and the second-generation product should be evaluated simultaneously. This can be accomplished by immobilizing each protein on separate channels in the microfluidic device and allowing serum samples to bind in series or in parallel to both immobilized proteins. The rationale for testing for binding...
to both the original and the modified therapeutic protein is that antibodies generated against the modified protein could have binding specificity to the original protein as well. As part of the characterization of the immune response, analysts must understand the specificity of antibodies for both first- and second-generation products. When possible, binding to an endogenous counterpart might also be tested by immobilizing the endogenous protein on a separate flow cell or channel.

**Characterization of Anti-Therapeutic Protein Antibodies:** Once antibodies against a therapeutic protein have been captured by binding to the immobilized therapeutic protein, those antibodies can be characterized. The important features of anti-therapeutic antibodies that can be studied include the relative binding affinity, the amount of antibodies present in the serum sample, the isotype(s) of antibodies present in the sample, and binding specificity.

By monitoring the rate at which the response units decrease after the conclusion of sample addition to the sensor, analysts can determine the relative affinity of the antibodies. A high rate of dissociation is characteristic of a low-affinity antibody, and a slow rate of dissociation suggests the presence of high-affinity antibodies. It is useful to compare the dissociation rates with both the positive control antibody (typically a high-affinity antibody preparation) as well as a panel of monoclonal antibodies of known binding affinities.

The relative active concentration of antibodies present in a sample can be estimated by comparing the binding signal with the signal produced from a dilution series of the positive control. Analysts can generate a standard curve from the standard and can compare the active concentration of antibodies in the sample with that standard curve. Because the positive control does not exactly mimic the mixture of antibodies contained in the sample—in fact, the positive control is often obtained from hyperimmunized animals such as rabbits—the concentration value obtained is relative to the standard. This value only approximates the actual concentration of human antibodies. Because the same positive control can be used throughout clinical development, analysts can compare the amount of antibodies obtained from different subjects using this strategy. Because the instrument’s signal is proportional to the mass that is binding to the sensor, this type of analysis provides value. Analysts should consider that IgM antibodies have five times the mass of IgG antibodies. Another approach for determining the concentration of antibodies is described in the concentration analysis section of this chapter (see Application 2 below).

The isotype of captured antibodies can be readily determined by monitoring binding associated with sequential addition of isotyping reagents. For example, if IgM antibodies are present and have bound to the immobilized protein, the addition of an anti-human IgM reagent will produce an additional signal. Isotyping reagents can be found with specificity towards IgM, IgG, IgE, IgA, IgG1, IgG2, IgG3, and IgG4. Because of steric hindrance, analysts may be required to repeat isotyping analyses in different sequences to be certain the presence of previously bound isotyping reagents has not hindered subsequent analyses. For example, assume a sample contains both IgG1 and IgG4 antibodies against a protein and both species have bound to the immobilized protein. Because the anti-IgG1 isotyping reagent has bound to the IgG1 antibodies, the isotyping reagent bound to them may prevent subsequent additions of an anti IgG4 reagent from binding to the IgG4, and the presence of the IgG4 would be undiscovered. Analysts will conclude that only IgG1 antibodies are present, but if the order of isotyping reagent addition were reversed the IgG4 antibodies would be discovered. This example underscores the importance of careful interpretation of isotyping results. This is a problem for subsequent analysis only if there is observed binding by a previous cycle of isotype reagent addition. The specificity of isotyping reagents should be confirmed before use. Analysts should, for example, verify that an anti-human IgG reagent binds only to human IgG and does not cross-react with human IgM.

The region of the therapeutic protein recognized by the antibodies can sometimes be determined by immobilizing versions of the protein that have been truncated, have point mutations, or contain only a fragment of the protein. If the antibodies fail to bind to the changed version of the protein, it suggests that the epitope toward which the antibody is directed was influenced by the change. It should be kept in mind that point mutations and truncations not only influence the primary sequence of a protein, but can also influence the tertiary structure (i.e. folding, conformation) of a protein. Also, a subject is likely to generate a population of antibodies with different specificities for a variety of epitopes. Despite this concern, the strategy just described can prove useful for identifying the region on the protein where the antibodies are binding.

**Application 2—Concentration Analysis:** SPR can be used to determine the concentration of biologics in defined buffer systems, e.g., eluates from purification columns, formulation buffers, and complex mixtures such as serum, fermentation broths, crude cell extracts, and cell suspensions. The concentration of an analyte is measured by its binding to the specific ligand or other molecules that can interact with any portion of the analyte. The analyte concentration is determined on a surface where the analyte-specific ligand or an analyte-specific capture reagent is immobilized. The binding rate or the mass of analyte bound is determined, and the analyte concentration is calculated using either a standard curve obtained from a concentration series of a purified and well characterized reference material or by a calibration-free analysis that is based on the relationship between the diffusion properties of the analyte and the absolute analyte concentration.

**Immobilization of Ligand:** To determine concentration the ligand is immobilized covalently or non-covalently on the surface. Analysts select an appropriate coupling mechanism and chemistry to ensure the ligand’s functional integrity. In order to provide conditions that favor partial or full independence of kinetic parameters, a high surface density of the ligand is desired.

A high-density surface allows the analyte to bind to the ligand under conditions that limit mass transport. The interaction between the analyte and the ligand can be described by the following two-step process:

\[
A \xrightarrow{k_a} A_{\text{surf}} + B \xrightarrow{k_+} AB
\]

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Concentration Determination with a Reference Standard Curve:

Calibration-free concentration assays are based on the relationship between the diffusion properties of the analyte and the absolute analyte concentration. By measuring the initial binding rate analysts can derive the analyte concentration if specific properties of analyte and the receptor are known. This approach can be useful when no satisfactory reference standard is available.

The ligand and the analyte reference material should be of sufficient purity with special attention to the presence of aggregated material. Aggregates of the analyte can interfere with the regeneration of the ligand surface because they can bind with multiple binding sites.

The reference material must be comparable (e.g., molecular weight and kinetic parameters) to the test samples. Under certain conditions the active concentration in unknown samples can be determined using a calibration-free procedure that is based on the relationship between the diffusion properties of the analyte and the absolute analyte concentration. These two methodologies are described separately below.

**Concentration Determination with a Reference Standard Curve:** In typical concentration-determination assays the analyte concentration is calculated from a standard curve that is obtained with a reference material injected at different concentrations. Three different approaches can be used to measure concentration with a reference standard calibration curve:

**Direct Binding Assay:** Determine the quantity of analyte bound after an arbitrarily fixed sample injection time. A sandwich method can be performed as an extension of the single-step direct binding approach in order to increase assay sensitivity.

**Binding Rate Determination:** Determine the initial binding rate for a sample rather than the amount bound. Under conditions of mass-transport limited binding, the binding rate is directly proportional to analyte concentration, and is independent of binding kinetics. This allows one to measure the concentration of related molecules that might have different binding characteristics.

**Inhibition or Competition Assays:** When the mechanism of action for an analyte is binding to a soluble ligand and thereby disrupting a ligand–receptor interaction, an inhibition assay can be used. In an inhibition assay, a receptor is attached to the sensor surface by a covalent linkage. The interaction between the analyte and the soluble ligand is indirectly measured by mixing a fixed concentration of ligand with varying concentrations of the analyte and injecting the ligand–analyte mixture across the immobilized receptor surface. Competitive methods in solution can also be used for large molecules and particles such as viruses, as well as for small analytes that give low direct responses. In parallel systems the assay can be designed so that the standard samples and the unknown sample are injected in parallel. This method can be useful for ligands that are difficult to regenerate.

Analysts can plot the signal (amount bound or rate of binding) of the reference material standards against concentrations and then can generate a standard curve using an appropriate mathematical model such as a linear or a logistic four-parameter curve fit. Samples can be injected at one or more dilutions. Fewer dilutions can be employed if a linear relationship between sample and reference standard has been demonstrated. Concentrations of unknown samples are either obtained by back-calculation from the standard curve or, if they are analyzed at the same target concentrations as those of the reference standard curve, by comparison of curve-fit parameters.

Parameters that can influence assay performance and results include but are not limited to flow rate, ligand density on the surface, sample purity, sample matrix, and reproducibility of surface regeneration. These parameters must be evaluated during assay qualification or validation. Interference with binding of analyte to the immobilized ligand can be minimized by salts, detergents, or sensor-surface support material. A commonly used sensor surface consists of carboxymethylated dextran, so the addition of dextran to the sample dilution buffer can minimize nonspecific interactions. Injections over a negative control surface can also be used to mathematically subtract the nonspecific binding data from the data obtained on the positive surface. A qualified or validated concentration determination SPR assay should include QC samples that can serve as measure to determine the accuracy of the standard curve that has been prepared to analyze samples with unknown analyte concentration. They can be conveniently prepared in larger batches, qualified for use with a Certificate of Analysis for the target concentration, and stored in small aliquots under appropriate storage conditions. After each analyte injection the ligand surface is regenerated and all bound analyte is removed. This regeneration must be strong enough to remove all bound analyte, but the conditions also must leave the immobilized ligand intact so that injections can be compared to each other.

**Concentration Determination Without Calibration:** Calibration-free concentration assays are based on the relationship between the diffusion properties of the analyte and the absolute analyte concentration. By measuring the initial binding rate analysts can derive the analyte concentration if specific properties of analyte and the analytical environment are known. This approach can be useful when no satisfactory reference standard is available.

To determine the analyte concentration in a sample, analysts use the relationship between initial binding rate and analyte concentration. On a sensor surface with a high immobilization level, the initial binding rate (slope) can be employed if a linear relationship between sample and reference standard has been demonstrated.
be described as a function of the molecular weight, the mass transport coefficient $k_m$, and the concentration of the analyte. Before a sample is analyzed analysts must determine the mass transport coefficient. It depends on the diffusion coefficient ($D$), flow rate, and flow cell dimensions and is described by the following formula:

$$k_m = 0.98 \times 10^{-5} \frac{D^2 \times f}{0.3 \times h^2 \times w \times l}$$

where $D$ is the diffusion coefficient, $f$ is the flow rate, and $h$, $w$, and $l$ are the flow cell height, width, and length, respectively. Flow rate and flow cell dimensions typically are known for a given instrument, and the diffusion coefficient is determined by the size and shape of the molecule by the use of instrument-specific tools, literature references, or experiments, e.g., by analytical ultracentrifugation or light scattering.

In a typical experimental setup the evaluation requires two flow rates. By using measurements at two widely separated flow rates, analysts can assess the influence of flow rate on binding rate. The robustness of the assay is also improved by fitting the data obtained at two different flow rates, which give correspondingly two different values for $k_m$ (because $k_m$ depends on the flow rate), to a model with a global variable for analyte concentration (so that the model is constrained to find a single concentration value that best fits both curves simultaneously).

Calibration-free concentration analysis is suitable only for proteins with MW $\geq$ 5000 Da. It requires fast analyte–ligand association ($k_a > 5 \times 10^4$ M$^{-1}$s$^{-1}$) and it cannot handle mixtures of analytes with different diffusion properties. The dynamic range of the method is approximately 0.05–5 µg/mL.

**Application 3—Kinetic and Affinity Analysis:** Because of its ability to detect binding interactions in real time, SPR provides valuable information about the kinetics of complex formation and dissociation. SPR instruments can be used to determine the association rate constant and dissociation rate constant for a particular binding interaction, and these values can be used subsequently to calculate the dissociation equilibrium constant ($K_d = k_d/k_a$). Obtaining $K_d$ from a ratio of $k_a$ and $k_d$ is useful when the binding interaction does not reach equilibrium in a timeframe that is suitable for an SPR binding experiment. For binding interactions that reach equilibrium (rate of complex formation equals the rate of complex decay) in min (vs. h), $K_d$ can be determined directly from a steady-state binding response.

The length of time required to reach equilibrium is influenced by the dissociation rate, so quickly dissociating complexes (e.g., $k_d = 10^{-5}$ s$^{-1}$) will reach equilibrium faster than those that dissociate slowly (e.g., $k_d = 10^{-3}$ s$^{-1}$).

Software programs capable of simulating 1:1 binding kinetics are useful for predicting the length of time required to reach equilibrium. The typical working range for affinity measurements with commercially available SPR instruments is from $10^{-15}$ M (pM) to $10^{-9}$ M (µM).

Proper experimental design is required to accurately measure $k_a$, $k_d$, and $K_d$. Several questions must be considered when designing kinetic analysis or steady-state affinity experiments, including:

- Which binding partner should be immobilized?
- How will the analyst immobilize one of the binding partners?
- What type of reference surface should be used?
- How much binding partner should be immobilized?
- Does the binding partner maintain activity after immobilization?
- Is binding to the immobilized binding partner specific?
- What regeneration conditions, if necessary, should be used?

When selecting which binding partner to immobilize for most protein–protein interactions, analysts must consider several factors: (1) the purity and availability of the proteins, (2) the presence of a tag or functional group to aid in immobilization, (3) maintaining biological activity, and (4) the binding valency (e.g., monovalent vs. multivalent binding).

A reference surface is required for all detailed kinetic and affinity analysis experiments. If direct immobilization is used, then a reference surface is created using the same immobilization protocol, omitting the protein during the coupling step. Alternatively, a mutant form of the protein with a modified binding site can be used. The reference surface for high-affinity capture typically consists of either the capture molecule and no binding partner, or uses an unrelated molecule for a mock capture surface. For the specific case of antibody–antigen interactions, an unrelated monoclonal antibody often serves as the capture reagent on the reference surface.

After deciding on the immobilization approach, analysts must decide how much binding partner to immobilize. For kinetic analysis, the primary consideration is to minimize the surface density to avoid mass-transport–limited binding of the analyte molecule to the immobilized binding partner. Analysts also must consider the immobilization level when conducting steady-state affinity analysis because high immobilization levels can cause steric hindrance or can induce secondary effects such as nonspecific binding or aggregation.

Before performing a kinetic experiment or steady-state affinity analysis, analysts must assess the activity of the surface by injecting the analyte molecule at a single concentration. The concentration should be high enough that the equilibrium binding response provides a close approximation of the experimental maximum response ($R_{max}$). This condition is typically met when the target molecule concentration is at least 10-fold higher than the $K_d$ of the binding interaction. For a protein–protein interaction having a $K_d$ value of 100 pM this means that the target molecule should be injected at a concentration of at least 1 nM. Using the $R_{max}$ equation ($Equations 1 and 2$), analysts can calculate the theoretical $R_{max}$ based on the amount of binding partner immobilized or captured. If the experimental $R_{max}$ exceeds the theoretical $R_{max}$ then the analyte molecule is larger than expected or the analyte exists in a higher-order structure than expected (e.g., following aggregation or as a multimer). If the experimental
**Use of SPR in a Regulated Environment**

When SPR assays are used for lot release and stability testing, the assay must exist within a controlled setting so that decisions can be made about the use of product within the clinic or marketplace. SPR instrumentation, including software, should be 21 CFR Part 11 compliant and should be amenable to validation. These requirements are important because they help ensure the integrity of both data acquisition and data evaluation.

Besides using SPR instrumentation that meets regulatory requirements, analysts should establish system suitability criteria for an SPR assay. Including these criteria in an SPR assay ensures that the results obtained for the test sample are generated by an assay that is performing within its operating parameters. A discussion of assessing system suitability parameters is not within the scope of this chapter, but the reader is referred to USP general chapters Design and Analysis of Biological Assays (111) and Analysis of Biological Assays (1034) for more detailed discussions. Some examples of system suitability parameters for an SPR assay can include:

- **ligand immobilization density**
- **parameters from a curve fitting model** (e.g., four parameter logistic curve fit)
The determination of the water activity of nonsterile pharmaceutical dosage forms aids in the decisions relating to the following:

1. optimizing product formulations to improve antimicrobial effectiveness of preservative systems,
2. reducing the degradation of active pharmaceutical ingredients within product formulations susceptible to chemical hydrolysis,
3. reducing the susceptibility of formulations (especially liquids, ointments, lotions, and creams) to microbial contamination, and
4. providing a tool for the rationale for reducing the frequency of microbial limit testing and screening for objectionable microorganisms for product release and stability testing using methods contained in the general test chapter Microbial Enumeration Tests (61) and Tests for Specified Microorganisms (62).

Reduced water activity ($a_w$) will greatly assist in the prevention of microbial proliferation in pharmaceutical products; and the formulation, manufacturing steps, and testing of nonsterile dosage forms should reflect this parameter.

Low water activity has traditionally been used to control microbial deterioration of foodstuffs. Examples where the available moisture is reduced are dried fruit, syrups, and pickled meats and vegetables. Low water activities make these materials self-preserved. Low water activity will also prevent microbial growth within pharmaceutical drug products. Other product attributes, for example, low or high pH, absence of nutrients, presence of surfactants, and addition of antimicrobial agents, as well as low water activity, help to prevent microbial growth. However, it should be noted that more resistant microorganisms, including spore-forming Clostridium spp., Bacillus spp., Salmonella spp. and filamentous fungi, although they may not proliferate in a drug product with a low water activity, may persist within the product.

When formulating an aqueous oral or topical dosage form, candidate formulations should be evaluated for water activity so that the drug product may be self-preserving, if possible. For example, small changes in the concentration of sodium chloride, sucrose, alcohol, propylene glycol, or glycerin in a formulation may result in the creation of a drug product with a lower water activity that can discourage the proliferation of microorganisms in the product. This is particularly valuable with a multiple-use product that may be contaminated by the user. Packaging studies should be conducted to test product stability and to determine that the container–closure system protects the product from moisture gains that would increase the water activity during storage.

Reduced microbial limits testing may be justified through risk assessment. This reduction in testing, when justified, may entail forgoing full microbial limits testing, implementing skip-lot testing, or eliminating routine testing. Nonaqueous liquids or dry solid dosage forms will not support spore germination or microbial growth due to their low water activity. The frequency of their microbial monitoring can be determined by a review of the historic testing database of the product and the demonstrated effectiveness of microbial contamination control of the raw materials, ingredient water, manufacturing process, formulation, and packaging system. The testing history would include microbial monitoring during product development, scale-up, process validation, and routine testing of sufficient marketed product lots (e.g., up to 20 lots) to ensure that the product has little or no potential for microbial contamination. Because the water activity requirements for
different Gram-reactive bacteria, bacterial spores, yeasts, and molds are well described in the literature,¹ the appropriate microbial limit testing program for products of differing water activities can be established. For example, Gram-negative bacteria including the specific objectionable microorganisms, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Salmonella* species will not proliferate or survive in preserved products with water activities below 0.91, while Gram-positive bacteria such as *Staphylococcus aureus* will not proliferate below 0.86, and *Aspergillus niger* will not proliferate below 0.77. Furthermore, even the most osmophilic yeast and xerophilic fungi will not proliferate below 0.60, and they cannot be isolated on compendial microbiological media.¹ The water activity requirements measured at 25° for the growth of a range of representative microorganisms are presented in Table 1.

### Table 1. Water Activities (a\textsubscript{w}) Required to Support the Growth of Representative Microorganisms

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Water Activity (a\textsubscript{w})</th>
<th>Molds and Yeast</th>
<th>Water Activity (a\textsubscript{w})</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>0.97</td>
<td><em>Rhizopus nigricans</em></td>
<td>0.93</td>
</tr>
<tr>
<td><em>Bacillus cereus</em></td>
<td>0.95</td>
<td><em>Mucor plumbeus</em></td>
<td>0.92</td>
</tr>
<tr>
<td><em>Clostridium botulinum, Type A</em></td>
<td>0.95</td>
<td><em>Rhodotorula mucilaginosa</em></td>
<td>0.92</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>0.95</td>
<td><em>Saccharomyces cerevisiae</em></td>
<td>0.90</td>
</tr>
<tr>
<td><em>Clostridium perfringens</em></td>
<td>0.95</td>
<td><em>Paeilomyces variotti</em></td>
<td>0.84</td>
</tr>
<tr>
<td><em>Lactobacillus viridescens</em></td>
<td>0.95</td>
<td><em>Penicillium chrysogenum</em></td>
<td>0.83</td>
</tr>
<tr>
<td><em>Salmonella</em> spp.</td>
<td>0.95</td>
<td><em>Aspergillus fumigatus</em></td>
<td>0.82</td>
</tr>
<tr>
<td><em>Enterobacter aerogenes</em></td>
<td>0.94</td>
<td><em>Penicillium glabrum</em></td>
<td>0.81</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>0.90</td>
<td><em>Aspergillus flavus</em></td>
<td>0.78</td>
</tr>
<tr>
<td><em>Micrococcus lysodeikticus</em></td>
<td>0.93</td>
<td><em>Aspergillus niger</em></td>
<td>0.77</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>0.86</td>
<td><em>Zygosachcharomyces rouxii</em> (osmophilic yeast)</td>
<td>0.62</td>
</tr>
<tr>
<td><em>Halobacterium halobium</em></td>
<td>0.75</td>
<td><em>Xeromyces bisporus</em> (xerophilic fungi)</td>
<td>0.61</td>
</tr>
</tbody>
</table>

Pharmaceutical drug products with water activities well below 0.75 (e.g., direct compression tablets, powder and liquid-filled capsules, nonaqueous liquid products, ointments, and rectal suppositories) would be excellent candidates for reduced microbial limit testing for product release and stability evaluation. This is especially true when pharmaceutical products are made from ingredients of good microbial quality, when manufacturing environments do not foster microbial contamination, when there are processes that inherently reduce the microbial content, when the formulation of the drug product has antimicrobial activity, and when manufacturing sites have an established testing history of low bioburden associated with their products. Table 2 contains suggested microbial limit testing strategies for typical pharmaceutical and over-the-counter (OTC) drug products based on water activity. Other considerations, as listed above, would be applied when setting up the microbial limits testing program for individual drug products because water activity measurements cannot solely be used to justify the elimination of microbial content testing for product release.

Similar arguments could be made for the microbial limits testing of pharmaceutical ingredients. However, this would require pharmaceutical manufacturers to have a comprehensive knowledge of the pharmaceutical ingredient manufacturer’s manufacturing processes, quality programs, and testing record. This could be obtained through a supplier audit program.

### Table 2. Microbial Limit Testing Strategy for Representative Pharmaceutical and OTC Drug Products Based on Water Activity

<table>
<thead>
<tr>
<th>Products</th>
<th>Water Activity (a\textsubscript{w})</th>
<th>Greatest Potential Contaminants</th>
<th>Testing Recommended</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nasal inhalant</td>
<td>0.99</td>
<td>Gram-negative bacteria</td>
<td>TAMC, TCYM, absence of <em>S. aureus</em> and <em>P. aeruginosa</em></td>
</tr>
<tr>
<td>Hair shampoo</td>
<td>0.99</td>
<td>Gram-negative bacteria</td>
<td>TAMC, TCYM, absence of <em>S. aureus</em> and <em>P. aeruginosa</em></td>
</tr>
<tr>
<td>Antacid</td>
<td>0.99</td>
<td>Gram-negative bacteria</td>
<td>TAMC, TCYM, absence of <em>E. coli</em> and <em>Salmonella</em> spp.</td>
</tr>
<tr>
<td>Topical cream</td>
<td>0.97</td>
<td>Gram-positive bacteria</td>
<td>TAMC, TCYM, absence of <em>S. aureus</em> and <em>P. aeruginosa</em></td>
</tr>
<tr>
<td>Oral liquid</td>
<td>0.90</td>
<td>Gram-positive bacteria and fungi</td>
<td>TAMC and TCYM</td>
</tr>
<tr>
<td>Oral suspension</td>
<td>0.87</td>
<td>Fungi</td>
<td>TAMC and TCYM</td>
</tr>
<tr>
<td>Topical ointment</td>
<td>0.55</td>
<td>None</td>
<td>Reduced testing</td>
</tr>
<tr>
<td>Lip balm</td>
<td>0.36</td>
<td>None</td>
<td>Reduced testing</td>
</tr>
<tr>
<td>Vaginal and rectal suppositories</td>
<td>0.30</td>
<td>None</td>
<td>Reduced testing</td>
</tr>
<tr>
<td>Compressed tablets</td>
<td>0.36</td>
<td>None</td>
<td>Reduced testing</td>
</tr>
</tbody>
</table>

Table 2. Microbial Limit Testing Strategy for Representative Pharmaceutical and OTC Drug Products Based on Water Activity (continued)

<table>
<thead>
<tr>
<th>Products</th>
<th>Water Activity ($a_w$)</th>
<th>Greatest Potential Contaminants</th>
<th>Testing Recommended</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liquid-filled capsule</td>
<td>0.30</td>
<td>None</td>
<td>Reduced testing</td>
</tr>
</tbody>
</table>

* TAMC = Total aerobic microbial count; TCYMC = Total combined yeast and mold count.

[Note—The water activities cited in Table 2 for the different dosage forms are representative, and companies are urged to test their individual products before developing a testing strategy.]

Water activity, $a_w$, is the ratio of vapor pressure of H$_2$O in product (P) to vapor pressure of pure H$_2$O (Po) at the same temperature. It is numerically equal to 1/100 of the relative humidity (RH) generated by the product in a closed system. RH can be calculated from direct measurements of partial vapor pressure or dew point or indirect measurement by sensors whose physical or electric characteristics are altered by the RH to which they are exposed.

The relationship between $a_w$ and equilibrium relative humidity (ERH) is represented by the following equations:

$$a_w = P/Po \text{ and } ERH(\%) = a_w \times 100$$

The $a_w$ measurement may be conducted using the dew point/chilled mirror method. A polished, chilled mirror is used as the condensing surface. The cooling system is electronically linked to a phototronic cell into which light is reflected from the condensing mirror. An air stream, in equilibrium with the test sample, is directed at the mirror which cools until condensation occurs on the mirror. The temperature at which this condensation begins is the dew point from which the ERH is determined. Sample preparation should be considered as it may affect the water activity level of the material tested. Commercially available instruments using the dew point/chilled mirror method or other technologies need to be evaluated for suitability, validated, and calibrated when used to make water activity determinations. These instruments are typically calibrated using saturated salt solutions at 25°C, as listed in Table 3.

Table 3. Standard Saturated Salt Solutions Used to Calibrate Water Activity Determination Instruments

<table>
<thead>
<tr>
<th>Saturated Salt Solutions</th>
<th>ERH (%)</th>
<th>$a_w$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potassium sulfate (K$_2$SO$_4$)</td>
<td>97.3</td>
<td>0.973</td>
</tr>
<tr>
<td>Barium chloride (BaCl$_2$)</td>
<td>90.2</td>
<td>0.902</td>
</tr>
<tr>
<td>Sodium chloride (NaCl)</td>
<td>75.3</td>
<td>0.753</td>
</tr>
<tr>
<td>Magnesium nitrate [Mg(NO$_3$)$_2$]</td>
<td>52.9</td>
<td>0.529</td>
</tr>
<tr>
<td>Magnesium chloride (MgCl$_2$)</td>
<td>32.8</td>
<td>0.328</td>
</tr>
</tbody>
</table>

**1116** MICROBIOLOGICAL CONTROL AND MONITORING OF ASEPTIC PROCESSING ENVIRONMENTS

Microbiologically controlled environments are used for a variety of purposes within the healthcare industry. This general information chapter provides information and recommendations for environments where the risk of microbial contamination is controlled through aseptic processing. Products manufactured in such environments include pharmaceutical sterile products, bulk sterile drug substances, sterile intermediates, excipients, and, in certain cases, medical devices. Aseptic processing environments are far more critical in terms of patient risk than controlled environments used for other manufacturing operations—for example, equipment and component preparation, limited bioburden control of nonsterile products, and processing of terminally sterilized products. In this chapter, the type of aseptic processing is differentiated by the presence or absence of human operators. An advanced aseptic process is one in which direct intervention with open product containers or exposed product contact surfaces by operators wearing conventional cleanroom garments is not required and never permitted. [Note—A description of terms used in this chapter can be found in the Glossary at the end of the chapter.]

The guidance provided in this chapter and the monitoring parameters given for microbiological evaluation should be applied only to clean rooms, restricted-access barrier systems (RABS), and isolators used for aseptic processing. ISO-classified environments used for other purposes are not required to meet the levels of contamination control required for aseptically produced sterile products. The environments used for nonsterile applications require different microbial control strategies.

A large proportion of products labeled as sterile are manufactured by aseptic processing rather than terminal sterilization. Because aseptic processing relies on the exclusion of microorganisms from the process stream and the prevention of microorganisms from entering open containers during processing, product bioburden as well as the bioburden of the manufacturing environment are important factors governing the risk of unacceptable microbial contamination. The terms aseptic and sterile are not synonymous. Sterile means having a complete absence of viable microorganisms or organisms that have the potential to reproduce. In the purest microbiological sense, an aseptic process is one that prevents contamination by the exclusion of microorganisms. In contemporary aseptic healthcare-product manufacturing, aseptic describes the process for

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handling sterilized materials in a controlled environment designed to maintain microbial contamination at levels known to present minimal risk.

In any environment where human operators are present, microbial contamination at some level is inevitable. Even the most cautious clean-room environment design and operation will not eliminate the shedding of microorganisms if human operators are present. Thus, an expectation of zero contamination at all locations during every aseptic processing operation is technically not possible and thus is unrealistic. There are no means to demonstrate that an aseptic processing environment and the product-contact surfaces within that environment are sterile. Monitoring locations should be determined based upon a significant period; and in each case, the contamination recovery rate metric should be established on the basis of a review of monitoring locations.

Environmental monitoring is usually performed by personnel and thus requires operator intervention. As a result, environmental monitoring can both increase the risk of contamination and also give false-positive results. Thus, intensive monitoring is unwarranted, particularly in the ISO 5 environments that are used in the most critical zones of aseptic processing.

A number of sampling methods can be used to assess and control the microbiological status of controlled environments for aseptic processing. At present, nearly all of these methods rely on the growth and recovery of microorganisms, many of which can be in a damaged state caused by environmental stress and therefore may be difficult to recover. The numerical values for microbial recovery and survival, and different air sampler suppliers may have designed their systems to give similar results under identical conditions, but rates of recovery have been reported to be both lower than expected and highly variable. In general, surface monitoring has been found to recover <50%, even when used with relatively high inoculum levels on standardized coupons. In actual production environments where organisms are stressed to varying degrees, recovery rates may be lower.

### ADVANCED ASEPTIC TECHNOLOGIES

Advanced aseptic technologies can be defined as those that do not rely on the direct intervention of human operators during processing. At present, technologies such as isolators, blow/fill/seal, and closed RABS (designs that are never opened during setup or operation) may be considered advanced aseptic technologies, provided that direct intervention by gowned personnel is disallowed during processing. In recent years, isolator technology has found a broad acceptance in healthcare manufacturing. Isolators and closed RABS effectively separate the operator from the critical aseptic processing environment. Because these systems substantially reduce contamination risk, their microbiological control levels are higher than those of conventional clean rooms that have comparable particulate air classification level, for example, ISO 5.

### CLEAN ROOM CLASSIFICATION FOR ASEPTIC PROCESSING ENVIRONMENTS

The design and construction of clean rooms and controlled environments are covered in ISO 14644 series. This standard describes the clean room classifications commonly used in the pharmaceutical industry. In aseptic processing, clean environments of ISO 14644-1 Classes 5–8 are typically used.
Isolators and closed RABS present a different picture, because personnel are excluded from the aseptic processing environment and manipulations are made using glove-and-sleeve assemblies and half-suits made of thick, flexible plastic (such as polyvinyl chloride or synthetic rubber). Personnel have far less effect on the microbial quality of the environment within an isolator enclosure than in clean room environments. Some users have chosen to operate RABS in a manner that allows open, direct human intervention. In an open operational state, these systems are more similar in operation to conventional clean rooms and therefore cannot be considered advanced aseptic processing systems. In an open RABS, the ability of operators to adversely affect microbial contamination risk is higher than with closed RABS or isolators.

Specifications for air changes per hour and air velocities are not included in ISO 14644, nor were they included in Federal Standard 209E. Typically, ISO Class 8/Class 100,000 rooms are designed to provide a minimum of 20 air changes per hour; ISO Class 7/Class 10,000 rooms are designed to provide more than 50 air changes per hour; and ISO Class 5/Class 100 clean rooms provide more than 100 air changes per hour. The design of some facility criteria may differ. By diluting and removing contaminants, large volumes of air are likely to reduce airborne contamination in aseptic production. Optimum conditions vary considerably, depending on process characteristics, particularly the amount of contamination derived from personnel. These specifications should be used only as a guide in the design and operation of clean rooms, because the precise correlations among air changes per hour, air velocity, and microbial control have not been satisfactorily established experimentally.

Manufacturers should maintain a predominantly unidirectional flow of air (either vertical or horizontal) in a staffed Class 5 clean room environment, particularly when products, product containers, and closures are exposed. In the evaluation of air movement within a clean room, studying airflow visually by smoke studies or other suitable means is probably more useful than using absolute measures of airflow velocity and change rates. Risk assessment models are another useful way of reducing contamination risk and should be considered.

Air velocity and change rates are far less important in isolators or closed RABS than in clean rooms because personnel are more carefully separated from the product, product containers, and closures. Air velocities substantially lower than those used in human-scale clean rooms have proved adequate in isolator systems and may be appropriate in RABS as well. In zones within isolators where particulate matter poses a hazard to product quality, predominantly vertical or horizontal unidirectional airflow can be maintained. Experience has shown that well-controlled mixing or turbulent airflow is satisfactory for many aseptic processes and for sterility testing within isolators (see Sterility Testing—Validation of Isolator Systems (1208)).

### IMPORTANCE OF A MICROBIOLOGICAL EVALUATION PROGRAM FOR CONTROLLED ENVIRONMENTS

Monitoring of total particulate count in controlled environments, even with the use of electronic instrumentation on a continuous basis, does not provide information on the microbiological content of the environment. The basic limitation of particulate counters is that they measure particles of 0.5 µm or larger. While airborne microorganisms are not free-floating or single cells, they frequently associate with particles of 10–20 µm. Particulate counts as well as microbial counts within controlled environments vary with the sampling location and the activities being conducted during sampling. Monitoring the environment for nonviable particulates and microorganisms is an important control function because they both are important in achieving product compliance requirements for Foreign and Particulate Matter and Sterility in Injections and Implanted Drug Products (1).

Total particulate monitoring may provide a better means of evaluating the overall quality of the environment in isolators and closed RABS than in most conventional clean rooms. The superior exclusion of human-borne contamination provided by an isolator results in an increased proportion of nonviable particulates. Total particulate counting in an isolator is likely to provide an immediate indicator of changes in contamination level. Microbial monitoring programs should assess the effectiveness of cleaning and sanitation practices by and of personnel who could have an impact on the bioburden. Because isolators are typically decontaminated using an automatic vapor or gas generation system, microbial monitoring is much less important in establishing their efficiency in eliminating bioburden. These automatic decontamination systems are validated directly, using an appropriate biological indicator challenge, and are controlled to defined exposure parameters during routine use to ensure consistent decontamination.

Microbial monitoring cannot and need not identify and quantify all microbial contaminants in these controlled environments. Microbiological monitoring of a clean room is technically a semiquantitative exercise, because a truly quantitative evaluation of the environment is not possible, given the limitations in sampling equipment. Both the lack of precision of enumeration methods and the restricted sample volumes that can be effectively analyzed suggest that environmental monitoring is incapable of providing direct quantitative information about sterility assurance. Analysts should remember that no microbiological sampling plan can prove the absence of microbial contamination, even when no viable contamination is recovered. The absence of growth on a microbiological sample means only that growth was not discovered; it does not mean that the environment is free of contamination.

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**Table 1. Airborne Total Particulate Cleanliness Classes**

<table>
<thead>
<tr>
<th>ISO Class&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Particles ≥0.5 µm/m&lt;sup&gt;3&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISO 5</td>
<td>3520</td>
</tr>
<tr>
<td>ISO 6</td>
<td>35,200</td>
</tr>
<tr>
<td>ISO 7</td>
<td>352,000</td>
</tr>
<tr>
<td>ISO 8</td>
<td>3,520,000</td>
</tr>
</tbody>
</table>

<sup>a</sup> Taken from ISO International Standard 14644 Part 1, published by the International Organization for Standardization, May 1999.

<sup>b</sup> The four ISO 14644-1 classes correspond closely to former U.S. Federal Standard 209E classifications. The relationships are ISO 5/Class 100, ISO 6/Class 1000, ISO 7/Class 10,000, and ISO 8/Class 100,000.
Routine microbial monitoring should provide sufficient information to demonstrate that the aseptic processing environment is operating in an adequate state of control. The real value of a microbiological monitoring program lies in its ability to confirm consistent, high-quality environmental conditions at all times. Monitoring programs can detect changes in the contamination recovery rate that may be indicative of changes in the state of control within the environment.

Environmental microbial monitoring and analysis of data by qualified personnel can assist in ensuring that a suitable state of control is maintained. The environment should be sampled during normal operations to allow the collection of meaningful, process-related data. Microbial sampling should occur when materials are in the area, processing activities are ongoing, and a full complement of personnel is working within the aseptic processing environment.

Microbial monitoring of manufacturing clean rooms, RABS, and isolators should include compressed gases, surfaces, room or enclosure air, and any other materials and equipment that might produce a risk of contamination. The analysis of contamination trends in an aseptic environment has long been a component of the environmental control program. In aseptic processing environments and particularly in ISO Class 5 environments, contamination is infrequently observed. In isolator enclosures, contamination is rarer still because of superior exclusion of human-borne contamination. Because of the criticality of these environments, even minor changes in the contamination incident rates may be significant, and manufacturers should frequently and carefully review monitoring data. In less critical environments, microbial contamination may be higher, but changes in recovery rates should be noted, investigated, and corrected. Isolated recoveries of microorganisms should be considered a normal phenomenon in conventional clean rooms, and these incidents generally do not require specific corrective action, because it is almost certain that investigations will fail to yield a scientifically verifiable cause. Because sampling itself requires an aseptic intervention in conventional clean rooms, any single uncorrelated contamination event could be a false positive.

When contamination recovery rates increase from an established norm, process and operational investigation should take place. Investigations will differ depending on the type and processing of the product manufactured in the clean room, RABS, or isolator. Investigation should include a review of area maintenance documentation; sanitization/decontamination documentation; the occurrence of nonroutine events; the inherent physical or operational parameters, such as changes in environmental temperature and relative humidity; and the training status of personnel.

In closed RABS and isolator systems, the loss of glove integrity or the accidental introduction of material that has not been decontaminated are among the most probable causes of detectable microbial contamination. Following the investigation, actions should be taken to correct or eliminate the most probable causes of contamination. Because of the relative rarity of contamination events in modern facilities, the investigation often proves inconclusive. When corrective actions are undertaken, they may include reinforcement of personnel training to emphasize acceptable gowning and aseptic techniques and microbial control of the environment. Some additional microbiological sampling at an increased frequency may be implemented, but this may not be appropriate during aseptic processing because intrusive or overly intensive sampling may entail an increased contamination risk. When additional monitoring is desirable, it may be more appropriate during process simulation studies. Other measures that can be considered to better control microbial contamination include additional sanitization, use of different sanitizing agents, and identification of the microbial contaminant and its possible source.

In any aseptic environment, conventional or advanced, the investigation and the rationale for the course of action chosen as a result of the investigation must be carefully and comprehensively documented.

**PHYSICAL EVALUATION OF CONTAMINATION CONTROL EFFECTIVENESS**

Clean environments should be certified as described in ISO 14644 series in order to meet their design classification requirements. The design, construction, and operation of clean rooms vary greatly, so it is difficult to generalize requirements for parameters such as filter integrity, air velocity, air patterns, air changes, and pressure differential. In particularly critical applications such as aseptic processing, a structured approach to physical risk assessment may be appropriate.

One such method has been developed by Ljundqvist and Reinmüller. This method, known as the L-R method, challenges the air ventilation system by evaluating both airflow and the ability of an environment to dilute and remove airborne particles. In the L-R method, a smoke generator allows analysts to visualize the air movements throughout a clean room or a controlled environment, including vortices or turbulent zones, and the airflow pattern can be fine-tuned to minimize these undesirable effects. Following visual optimization of airflow, particulate matter is generated close to the critical zone and sterile field. This evaluation is done under simulated production conditions but with equipment and personnel in place. This type of test can also be used to evaluate the ability of RABS and isolator systems, particularly around product exit ports in these systems, to resist the effects of contamination.

Visual evaluation of air movement within clean rooms is a subjective process. Complete elimination of turbulence or vortices is not possible in operationing clean rooms that contain personnel and equipment. Air visualization is simply one step in the effort to optimize clean room operations and is not a definitive pass/fail test, because acceptable or unacceptable conditions are not readily definable.

Proper testing and optimization of the physical characteristics of the clean room, RABS, or isolator are essential before implementation of the microbiological monitoring program. Assurance that the clean room, RABS, or isolator is in compliance with its predetermined engineering specifications provides confidence that the ability of the facility systems and operating practices to control the bioburden and nonviable particulate matter are appropriate for the intended use. These tests should be repeated during routine certification of the clean room or advanced aseptic processing systems, and whenever significant changes are made to the operation, such as personnel flow, equipment operation, material flow, air-handling systems, or equipment layout.
TRAINING OF PERSONNEL

Good personnel performance plays an essential role in the control of contamination, proper training and supervision are central to contamination control. Aseptic processing is the most critical activity conducted in microbiological controlled environments, and manufacturers must pay close attention to details in all aspects of this endeavor. Rigorous discipline and strict supervision of personnel are essential in order to ensure a level of environmental quality appropriate for aseptic processing.

Training of all personnel working in controlled environments is critical. This training is equally important for personnel responsible for the microbial monitoring program, because contamination of the clean working area could inadvertently occur during microbial sampling. In highly automated operations, monitoring personnel may be the employees who have the most direct contact with the critical surfaces and zones within the processing area. Microbiological sampling has the potential to contribute to microbial contamination caused by inappropriate sampling techniques or by placing personnel in or near the critical zone. A formal training program is required to minimize this risk. This training should be documented for all personnel who enter controlled environments. Interventions should always be minimized, including those required for monitoring activities; but when interventions cannot be avoided, they must be conducted with aseptic technique that approaches perfection as closely as possible.

Management of the facility must ensure that personnel involved in operations in clean rooms and advanced aseptic processing environments are well versed in relevant microbiological principles. The training should include instruction about the basic principles of aseptic technique and should emphasize the relationship of manufacturing and handling procedures to potential sources of product contamination. Those supervising, auditing, or inspecting microbiological control and monitoring activities should be knowledgeable about the basic principles of microbiology, microbial physiology, disinfection and sanitation, media selection and preparation, taxonomy, and sterilization. The staff responsible for supervision and testing should have academic training in medical or environmental microbiology. Sampling personnel as well as individuals working in clean rooms should be knowledgeable about their responsibilities in minimizing the release of microbial contamination. Personnel involved in microbial identification require specialized training about required laboratory methods. Additional training about the management of collected data must be provided. Knowledge and understanding of applicable standard operating procedures are critical, especially those procedures relating to corrective measures taken when environmental conditions require.

Understanding of contamination control principles and each individual’s responsibilities with respect to good manufacturing practices (GMPs) should be an integral part of the training program, along with training in conducting investigations and in analyzing data.

The only significant sources of microbial contamination in aseptic environments are the personnel. Because operators disperse contamination and because the ultimate objective in aseptic processing is to reduce end-user risk, only healthy individuals should be permitted access to controlled environments. Individuals who are ill must not be allowed to enter an aseptic processing environment, even one that employs advanced aseptic technologies such as isolators, blow/fill/seal, or closed RABS.

The importance of good personal hygiene and a careful attention to detail in aseptic gowning cannot be overemphasized. Gowning requirements differ depending on the use of the controlled environment and the specifics of the gowning system itself. Aseptic processing environments require the use of sterilized gowns with the best available filtration properties. The fullest possible skin coverage is desirable, and sleeve covers or tape should be considered to minimize leaks at the critical glove–sleeve junction. Exposed skin should never be visible in conventional clean rooms under any conditions. The personnel and gowning considerations for RABS are essentially identical to those for conventional clean rooms.

Once employees are properly gowned, they must be careful to maintain the integrity of their gloves, masks, and other gown materials at all times. Operators who work with isolator systems are not required to wear sterilized clean-room gowns, but inadequate aseptic technique and employee-borne contamination are the principal hazards to safe aseptic operations in isolators, as well as RABS, and in conventional clean rooms. Glove-and-sleeve assemblies can develop leaks that can allow the mechanical transfer of microorganisms to the product. A second glove, worn either under or over the primary isolator/RABS glove, can provide an additional level of safety against glove leaks or can act as a hygienic measure. Also, operators must understand that aseptic technique is an absolute requirement for all manipulations performed with gloves within RABS and isolator systems.

The environmental monitoring program, by itself, cannot detect all events in aseptic processing that might compromise the microbiological quality of the environment. Therefore, periodic media-fill or process simulation studies are necessary, as is thorough ongoing supervision, to ensure that appropriate operating controls and training are effectively maintained.

CRITICAL FACTORS IN THE DESIGN AND IMPLEMENTATION OF A MICROBIOLOGICAL ENVIRONMENTAL MONITORING PROGRAM

Since the advent of comprehensive environmental monitoring programs, their applications in capturing adverse trends or drifts has been emphasized. In a modern aseptic processing environment—whether an isolator, RABS, or conventional clean room—contamination has become increasingly rare. Nevertheless, a monitoring program should be able to detect a change from the validated state of control in a facility and to provide information for implementing appropriate countermeasures. An environmental monitoring program should be tailored to specific facilities and conditions. It is also helpful to take a broad perspective in the interpretation of data. A single uncorrelated result on a given day may not be significant in the context of the technical limitations associated with aseptic sampling methods.

Selection of Growth Media

A general microbiological growth medium such as soybean–casein digest medium (SCDM) is suitable for environmental monitoring in most cases because it supports the growth of a wide range of bacteria, yeast, and molds. This medium can be supplemented with additives to overcome or to minimize the effects of sanitizing agents or of antibiotics. Manufacturers should
consider the specific detection of yeasts and molds. If necessary, general microbiological growth media such as Sabouraud’s, modified Sabouraud’s, or inhibitory mold agar can be used. In general, monitoring for strict anaerobes is not performed, because these organisms are unlikely to survive in ambient air. However, micro-aerophilic organisms may be observed in aseptic processing. Should anoxic conditions exist or if investigations warrant (e.g., identification of these organisms in sterility testing facilities or Sterility Tests (71) results), monitoring for micro-aerophiles and organisms that grow under low-oxygen conditions may be warranted. The ability of any media used in environmental monitoring, including those selected to recover specific types of organisms, must be evaluated for their ability to support growth, as indicated in (71).

**Selection of Culture Conditions**

Time and incubation temperatures are set once the appropriate media have been selected. Typically, for general microbiological growth media such as SCDM, incubation temperatures in the ranges of approximately 20°–35° have been used with an incubation time of not less than 72 hours. Longer incubation times may be considered when contaminants are known to be slow growing. The temperature ranges given above are by no means absolute. Mesophilic bacteria and mold common to the typical facility environment are generally capable of growing over a wide range of temperatures. For many mesophilic organisms, recovery is possible over a range of approximately 20°. In the absence of confirmatory evidence, microbiologists may incubate a single plate at both a low and a higher temperature. Incubating at the lower temperature first may compromise the recovery of Gram-positive cocci that are important because they are often associated with humans.

Sterilization processes for preparing growth media should be validated. When selective media are used for monitoring, incubation conditions should reflect published technical requirements. Contamination should not be introduced into a manufacturing clean room as a result of using contaminated sampling media or equipment. Of particular concern is the use of aseptically prepared sampling media. Wherever possible, sampling media and their wrappings should be terminally sterilized by moist heat, radiation, or other suitable means. If aseptically prepared media must be used, analysts must carry out preincubation and visual inspection of all sampling media before introduction into the clean room. The reader is referred to Microbiological Best Laboratory Practices (1117) for further information regarding microbiology laboratory operations and control.

**ESTABLISHMENT OF SAMPLING PLAN AND SITES**

During initial startup or commissioning of a clean room or other controlled environment, specific locations for air and surface sampling should be determined. Locations considered should include those in proximity of the exposed product, containers, closures, and product contact surfaces. In aseptic processing, the area in which containers, closures, and product are exposed to the environment is often called the critical zone—the critical zone is always ISO 5. For aseptic operations the entire critical zone should be treated as a sterile field. A nonsterile object, including the gloved hands of clean room personnel or an RABS/isolator glove, should never be brought into contact with a sterile product, container closure, filling station, or conveying equipment before or during aseptic processing operations. Operators and environmental monitoring personnel should never touch sterile parts of conveyors, filling needles, parts hoppers, or any other equipment that is in the product-delivery pathway. This means that surface monitoring on these surfaces is best done at the end of production operations.

The frequency of sampling depends on the manufacturing process conducted within an environment. Classified environments that are used only to provide a lower overall level of bioburden in nonsterile product manufacturing areas require relatively infrequent environmental monitoring. Classified environments in which closed manufacturing operations are conducted, including fermentation, sterile API processing, and chemical processes, require fewer monitoring sites and less frequent monitoring because the risk of microbial contamination from the surrounding environment is comparatively low. Microbiological monitoring of environments in which products are filled before terminal sterilization is generally less critical than the monitoring of aseptic processing areas. The amount of monitoring required in filling operations for terminal sterilization depends on the susceptibility of the product survival and the potential for proliferation of microbial contamination. The identification and estimated number of microorganisms that are resistant to the subsequent sterilization may be more critical than the microbiological monitoring of the surrounding manufacturing environments.

It is not possible to recommend microbial control levels for each type of manufacturing environment. The levels established for one ISO Class 7 environment, for example, may be inappropriate for another ISO Class 7 environment, depending on the production activities undertaken in each. The user should conduct a prospective risk analysis and develop a rationale for the sampling locations and frequencies for each controlled environment. The classification of a clean room helps establish control levels, but that does not imply that all rooms of the same classification should have the same control levels and the same frequency of monitoring. Monitoring should reflect the microbiological control requirements of manufacturing or processing activities. Formal risk assessment techniques can result in a scientifically valid contamination control program.

**Table 2. Suggested Frequency of Sampling for Aseptic Processing Areas**

<table>
<thead>
<tr>
<th>Sampling Area/Location</th>
<th>Frequency of Sampling</th>
</tr>
</thead>
<tbody>
<tr>
<td>Critical zone (ISO 5 or better)</td>
<td>Clean Room/RABS</td>
</tr>
</tbody>
</table>

Published on March 26, 2020
TABLE 2. SUGGESTED FREQUENCY OF SAMPLING FOR ASEPTIC PROCESSING AREAS* (continued)

<table>
<thead>
<tr>
<th>Sampling Area/Location</th>
<th>Frequency of Sampling</th>
</tr>
</thead>
<tbody>
<tr>
<td>Active air sampling</td>
<td>Each operational shift</td>
</tr>
<tr>
<td>Surface monitoring</td>
<td>At the end of the operation</td>
</tr>
<tr>
<td>Aseptic area adjacent critical zone</td>
<td>Each operating shift</td>
</tr>
<tr>
<td>All sampling</td>
<td>Once per day</td>
</tr>
<tr>
<td>Other nonadjacent aseptic areas</td>
<td>Isolators</td>
</tr>
<tr>
<td>Critical zone (ISO 5 or better)</td>
<td>Once per day</td>
</tr>
<tr>
<td>Active air sampling</td>
<td>Isolators</td>
</tr>
<tr>
<td>Surface monitoring</td>
<td>At the end of the campaign</td>
</tr>
<tr>
<td>Nonaseptic areas surrounding the isolator</td>
<td>Once per month</td>
</tr>
</tbody>
</table>

*All operators are aseptically gowned in these environments (with the exception of background environments for isolators). These recommendations do not apply to production areas for nonsterile products or other classified environments in which fully aseptic gowns are not donned.

**SELECTIVE CONTROL PARAMETERS IN CLEAN ROOMS, ISOLATORS, AND RABS**

ISO 14644 suggests a grid approach for the total particulate air classification of clean rooms. This approach is appropriate for certifying the total particulate air quality performance against its design objective. Grids may also have value in analyzing risk from microbial contamination, although in general, grids that have no personnel activity are likely to have low risk of contamination. Microbial contamination is strongly associated with personnel, so microbiological monitoring of unstaffed environments is of limited value.

Microbiological sampling sites are best selected with consideration of human activity during manufacturing operations. Careful observation and mapping of the clean room during the qualification phase can provide useful information concerning the movement and positioning of personnel. Such observation can also yield important information about the most frequently conducted manipulations and interventions.

The location and movement of personnel within the clean room correlate with contamination risk to the environment and to the processes conducted within that environment. Sample sites should be selected so that they evaluate the impact of personnel movement and work within the area, particularly interventions and manipulations within the critical zone.

The most likely route of contamination is airborne, so the samples most critical to risk assessment are those that relate to airborne contamination near exposed sterile materials. Other areas of concern are entry points where equipment and materials move from areas of lower classification to those of higher classification. Areas within and around doors and airlocks should be included in the monitoring scheme. It is customary to sample walls and floors, and indeed sampling at these locations can provide information about the effectiveness of the sanitation program. Sampling at these locations can take place relatively infrequently, because contamination there is unlikely to affect product. Operators should never touch floors and walls, so mechanical transmission of contamination from these surfaces to critical areas where product is exposed should not occur.

Manufacturers typically monitor surfaces within the critical zone, although this should be done only at the end of operations. Residues of media or diluent from wet swabs should be avoided on surfaces, because they could lead to microbial proliferation.

Cleaning surfaces to remove diluent or media requires personnel intervention and movements that can result in release of microbial contamination into the critical zone and can disrupt airflow.

**MICROBIOLOGICAL CONTROL PARAMETERS IN CLEAN ROOMS, ISOLATORS, AND RABS**

Since the early 1980s, manufacturers have established alert and action levels for environmental monitoring. In recent years the numerical difference between alert and action levels has become quite small, especially in ISO 5 environments. Growth and recovery in microbiological assays have normal variability in the range of ±0.5 log_{10}. Studies on active microbiological air samplers indicate that variability of as high as tenfold is possible among commonly used sampling devices. As a result of this inherent variability and indeterminate sampling error, the supposed differences between, for example, an alert level of 1 cfu and an action level of 3 cfu are not analytically significant. Treating differences that are within expected, and therefore, normal ranges as numerically different is not scientifically valid and can result in unwarranted activities. In a practical sense, numerical values that vary by as much as five- to tenfold may not be significantly different.

Because of the limited accuracy and precision of microbial growth and recovery assays, analysts can consider the frequency with which contamination is detected rather than absolute numbers of cfu detected in any single sample. Also, a cfu is not a direct enumeration of microorganisms present but rather is a measure of contamination that may have originated from a clump of organisms.

Mean contamination recovery rates should be determined for each clean room environment, and changes in contamination recovery rate at a given site or within a given room may indicate the need for corrective action. Within the ISO 5 critical zone, airborne and surface contamination recovery rates of 1% or less should be attainable with current methods.
recovery rates for closed RABS and isolator systems should be significantly lower still and can be expected to be <0.1%, on the basis of published monitoring results.

Contamination observed at multiple sites in an environment within a single sampling period may indicate increased risk to product and should be carefully evaluated. The appearance of contamination nearly simultaneously at multiple sites could also arise from poor sampling technique, so careful review is in order before drawing conclusions about potential loss of control. Resampling an environment several days after contamination is of little value, because the conditions during one sampling occasion may not be accurately duplicated during another.

Surface samples may also be taken from clean room garments. Personnel sampling should be emphasized during validation and is best done at the completion of production work in order to avoid adventitious contamination of the garments. In this case the average should be <1% for these sample sites as well. Gloves on closed RABS and isolators should meet the more rigorous expectation of <0.1% contamination recovery rates.

Because of the inherent variability of microbial sampling methods, contamination recovery rates are a more useful measure of trending results than is focusing on the number of colonies recovered from a given sample. Table 3 provides recommended contamination recovery rates for aseptic processing environments. The incident rate is the rate at which environmental samples are found to contain microbial contamination. For example, an incident rate of 1% would mean that only 1% of the samples taken have any contamination regardless of colony number. In other words, 99% of the samples taken are completely free of contamination. Contamination recovery rates that are higher than those recommended in Table 3 may be acceptable in rooms of similar classification that are used for lower-risk activities. Action should be required when the contamination recovery rate trends above these recommendations for a significant time.

### Table 3. Suggested Initial Contamination Recovery Rates in Aseptic Environments*

<table>
<thead>
<tr>
<th>Room Classification</th>
<th>Active Air Sample (%</th>
<th>Settle Plate (9 cm) 4 h Exposure (%)</th>
<th>Contact Plate or Swab (%)</th>
<th>Glove or Garment (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolator/Closed RABS (ISO 5 or better)</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>ISO 5</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>ISO 6</td>
<td>&lt;3</td>
<td>&lt;3</td>
<td>&lt;3</td>
<td>&lt;3</td>
</tr>
<tr>
<td>ISO 7</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>ISO 8</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
</tbody>
</table>

*All operators are aseptically gowned in these environments (with the exception of background environments for isolators). These recommendations do not apply to production areas for nonsterile products or other classified environments in which fully aseptic gowns are not donned.

Detection frequency should be based on actual monitoring data and should be retabulated monthly. Action levels should be based on empirical process capability. If detection frequencies exceed the recommendations in Table 3 or are greater than established process capability, then corrective actions should be taken. Corrective actions may include but are not limited to the following:

- Revision of the sanitization program, including selection of antimicrobial agents, application methods, and frequencies
- Increased surveillance of personnel practices, possibly including written critiques of aseptic methods and techniques
- Review of microbiological sampling methods and techniques

When higher-than-typical recovery levels for glove and garment contamination are observed, additional training for gowning practices may be indicated.

### SIGNIFICANT EXCURSIONS

Excursions beyond approximately 15 cfu recovered from a single ISO 5 sample, whether from airborne, surface, or personnel sources, should happen very infrequently. When such ISO 5 excursions do occur, they may be indicative of a significant loss of control when they occur within the ISO 5 critical zone in close proximity to product and components. Thus, any ISO 5 excursion >15 cfu should prompt a careful and thorough investigation.

A key consideration for an abnormally high number of recovered colonies is whether this incident is isolated or can be correlated with other recoveries. Microbiologists should review recovery rates for at least two weeks before the incident of abnormally high recovery so that they can be aware of other recoveries that might indicate an unusual pattern. Microbiologists should carefully consider all recoveries, including those that are in the more typical range of 1–5 cfu. The identity of the organisms recovered is an important factor in the conduct of this investigation.

In the case of an isolated single excursion, establishing a definitive cause probably will not be possible, and only general corrective measures can be considered. It is never wise to suggest a root cause for which there is no solid scientific evidence. Also, there should be an awareness of the variability of microbial analysis. Realistically, there is no scientific reason to treat a recovery of 25 cfu as statistically different from a recovery of 15 cfu. A value of 15 cfu should not be considered significant in terms of process control, because realistically there is no difference between a recovery of 14 cfu and one of 15 cfu. Microbiologists should use practical scientific judgment in their approach to excursions.

### FURTHER CONSIDERATIONS ABOUT DATA INTERPRETATION

In the high-quality environments required for aseptic processing, detection frequency typically is low. As can be seen from the rates recommended in Table 3, the majority of samples taken in an aseptic processing area will yield a recovery of zero.
contamination. In the most critical areas within an aseptic processing operation, it is expected that less than 1% of the samples will yield any recoverable contamination. In the most advanced of modern aseptic operations that use separative technologies such as isolators or closed RABS, the recovery rate will approach zero at all times.

The microbiologist responsible for environmental control or sterility assurance should not take this to mean that the environmental quality approaches sterility. The sensitivity of any microbial sampling system in absolute terms is not known. In environmental monitoring, a result of zero means only that the result is below the limit of detection of the analytical system. A false sense of security should not be derived from the infrequency of contamination recovery in aseptic processing.

Sterility assurance is best accomplished by a focus on human-borne contamination and the facility design features that best mitigate risk from this contamination. Greatest risk mitigation can be attained by reducing or eliminating human interventions through proper equipment design and by providing sufficient air exchanges per hour for the intended personnel population of the facility. Other risk mitigation factors include effective personnel and material movement and the proper control of temperature and humidity. Secondary factors for risk mitigation include cleaning and sanitization. Risk analysis models that analyze processes prospectively to reduce human-borne contamination risk by minimizing operator interventions are more powerful tools for sterility assurance than monitoring. Environmental monitoring cannot prove or disprove in absolute terms the sterility of a lot of product. Environmental monitoring can only assure those responsible for a process that a production system is in a consistent, validated state of control. Care should be taken to avoid drawing inappropriate conclusions from monitoring results.

**SAMPLING AIRBORNE MICROORGANISMS**

Among the most commonly used tools for monitoring aseptic environments are impaction and centrifugal samplers. A number of commercially available samplers are listed for informational purposes. The selection, appropriateness, and adequacy of using any particular sampler are the responsibility of the user.

**Slit-to-Agar Air Sampler (STA)**

The unit is powered by an attached source of controllable vacuum. The air intake is obtained through a standardized slit below which is placed a slowly revolving Petri dish that contains a nutrient agar. Airborne particles that have sufficient mass impact the agar surface, and viable organisms are allowed to grow. A remote air intake is often used to minimize disturbance of unidirectional airflow.

**Sieve Impactor**

This apparatus consists of a container designed to accommodate a Petri dish that contains a nutrient agar. The cover of the unit is perforated with openings of a predetermined size. A vacuum pump draws a known volume of air through the cover, and airborne particles that contain microorganisms impact the agar medium in the Petri dish. Some samplers feature a cascaded series of sieves that contain perforations of decreasing size. These units allow determination of the size range distribution of particulates that contain viable microorganisms based on the size of the perforations through which the particles landed on the agar plates.

**Centrifugal Sampler**

The unit consists of a propeller or turbine that pulls a known volume of air into the unit and then propels the air outward to impact on a tangentially placed nutrient agar strip set on a flexible plastic base.

**Sterilizable Microbiological Atrium**

The unit is a variant of the single-stage sieve impactor. The unit’s cover contains uniformly spaced orifices approximately 0.25 inch in size. The base of the unit accommodates one Petri dish containing a nutrient agar. A vacuum pump controls the movement of air through the unit, and a multiple-unit control center as well as a remote sampling probe are available.

**Surface Air System Sampler**

This integrated unit consists of an entry section that accommodates an agar contact plate. Immediately behind the contact plate is a motor and turbine that pulls air through the unit’s perforated cover over the agar contact plate and beyond the motor, where it is exhausted. Multiple mounted assemblies are also available.

**Gelatin Filter Sampler**

The unit consists of a vacuum pump with an extension hose terminating in a filter holder that can be located remotely in the critical space. The filter consists of random fibers of gelatin capable of retaining airborne microorganisms. After a specified exposure time, the filter is aseptically removed and dissolved in an appropriate diluent and then plated on an appropriate agar medium to estimate its microbial content.
Settling Plates

This method is still widely used as a simple and inexpensive way to qualitatively assess the environments over prolonged exposure times. Published data indicate that settling plates, when exposed for 4- to 5-hour periods, can provide a limit of detection for a suitable evaluation of the aseptic environment. Settling plates may be particularly useful in critical areas where active sampling could be intrusive and a hazard to the aseptic operation.

One of the major drawbacks of mechanical air samplers is the limited sample size of air being tested. When the microbial level in the air of a controlled environment is expected to contain extremely low levels of contamination per unit volume, at least 1 cubic meter of air should be tested in order to maximize sensitivity. Typically, slit-to-agar devices have an 80-L/min sampling capacity (the capacity of the surface air system is somewhat higher). If 1 cubic meter of air were tested, then it would require an exposure time of 15 min. It may be necessary to use sampling times in excess of 15 min to obtain a representative environmental sample. Although some samplers are reported to have high sampling volumes, consideration should be given to the potential for disruption of the airflow patterns in any critical area and to the creation of turbulence.

Technicians may wish to use remote sampling systems in order to minimize potential risks resulting from intervention by environmental samplers in critical zones. Regardless of the type of sampler used, analysts must determine that the extra tubing needed for a remote probe does not reduce the method’s sensitivity to such an extent that detection of low levels of contamination becomes unlikely or even impossible.

SURFACE SAMPLING

Another component of the microbial-control program in controlled environments is surface sampling of equipment, facilities, and personnel. The standardization of surface sampling methods and procedures has not been as widely addressed in the pharmaceutical industry as has the standardization of air-sampling procedures. Surface sampling can be accomplished by the use of contact plates or by the swabbing method.

Contact plates filled with nutrient agar are used for sampling regular or flat surfaces and are directly incubated for the appropriate time and temperature for recovery of viable organisms. Specialized agar can be used for the recovery of organisms that have specific growth requirements. Microbial estimates are reported per contact plate.

The swabbing method can be used to supplement contact plates for sampling of irregular surfaces, especially irregular surfaces of equipment. The area that will be swabbed is defined with a sterile template of appropriate size. In general, it is in the range of 24–30 cm². After sample collection the swab is placed in an appropriate diluent or transport medium and is plated onto the desired nutrient agar. The microbial estimates are reported per swab of defined sampling area.

Surface monitoring is used as an environmental assessment tool in all types of classified environments. In ISO 5 environments for aseptic processing, surface monitoring is generally performed beside critical areas and surfaces. Component hoppers and feed chutes that contact sterile surfaces on closures and filling needles can be tested for microbial contamination. Often in conventional staffed clean rooms, these product contact surfaces are steam sterilized and aseptically assembled. The ability of operators to perform these aseptic manipulations are evaluated during process stimulations or media fills, although true validation of operator technique in this manner is not possible. Surface monitoring on surfaces that directly contact sterile parts or product should be done only after production operations are completed. Surface sampling is not a sterility test and should not be a criterion for the release or rejection of product. Because these samples must be taken aseptically by personnel, it is difficult to establish with certainty that any contamination recovered is product related.

CULTURE MEDIA AND DILUENTS

The type of medium, liquid or solid, used for sampling or plating microorganisms depends on the procedure and equipment used. Any medium used should be evaluated for suitability for the intended purpose. The most commonly used all-purpose solid microbiological growth medium is soybean–casein digest agar. As previously noted, this medium can be supplemented with chemicals that counteract the effect of various antimicrobials.

IDENTIFICATION OF MICROBIAL ISOLATES

A successful environmental control program includes an appropriate level of identification of the flora obtained by sampling. A knowledge of the flora in controlled environments aids in determining the usual microbial flora anticipated for the facility and in evaluating the effectiveness of the cleaning and sanitization procedures, methods, agents, and recovery methods. The information gathered by an identification program can be useful in the investigation of the source of contamination, especially when recommended detection frequencies are exceeded.

Identification of isolates from critical and immediately adjacent areas should take precedence over identification of microorganisms from noncritical areas. Identification methods should be verified, and ready-to-use kits should be qualified for their intended purpose.

CONCLUSION

Environmental monitoring is one of several key elements required in order to ensure that an aseptic processing area is maintained in an adequate level of control. Monitoring is a qualitative exercise, and even in the most critical applications such as aseptic processing, conclusions regarding lot acceptability should not be made on the basis of environmental sampling results alone. Environments that are essentially free of human operators generally have low initial contamination rates and maintain...
low levels of microbial contamination. Human-scale clean rooms present a very different picture. Studies conclusively show that operators, even when carefully and correctly gowned, continuously slough microorganisms into the environment. Therefore, it is unreasonable to assume that samples produced no colonies, even in the critical zone or on critical surfaces, will always be observed. Periodic excursions are a fact of life in human-scale clean rooms, but the contamination recovery rate, particularly in ISO 5 environments used for aseptic processing, should be consistently low.

Clean-room operators, particularly those engaged in aseptic processing, must strive to maintain suitable environmental quality and must work toward continuous improvement of personnel operations and environmental control. In general, fewer personnel involved in aseptic processing and monitoring, along with reduction in interventions, reduces risk from microbial contamination.

GLOSSARY

**Aseptic**: Technically, the absence of microorganisms, but in aseptic processing this refers to methods and operations that minimize microbial contamination in environments where sterilized product and components are filled and/or assembled.

**Aseptic Processing**: An operation in which the product is assembled or filled into its primary package in an ISO 5 or better environment and under conditions that minimize the risk of microbial contamination. The ultimate goal is to produce products that are as free as possible of microbial contamination.

**Barrier System**: Physical barriers installed within an aseptic processing room to provide partial separation between aseptically gownned personnel and critical areas subject to considerable contamination risk. Personnel access to the critical zone is largely unrestricted. It is subject to a high level disinfection.

**Bioburden**: Total number and identity of the predominant microorganisms detected in or on an article.

**Clean Room**: A room in which the concentration of airborne particles is controlled to meet a specified airborne particulate cleanliness Class. In addition, the concentration of microorganisms in the environment is monitored; each cleanliness Class defined is also assigned a microbial level for air, surface, and personnel gear.

**Commissioning of a Controlled Environment**: Certification by engineering and quality control that the environment has been built according to the specifications of the desired cleanliness Class and that, under conditions likely to be encountered under normal operating conditions (or worst-case conditions), it is capable of delivering an aseptic process. Commissioning includes media-fill runs and results of the environmental monitoring program.

**Contamination Recovery Rate**: The contamination recovery rate is the rate at which environmental samples are found to contain any level of contamination. For example, an incident rate of 1% would mean that only 1% of the samples taken have any contamination regardless of colony number.

**Controlled Environment**: Any area in an aseptic process system for which airborne particulate and microorganism levels are controlled to specific levels, appropriate to the activities conducted within that environment.

**Corrective Action**: Actions to be performed that are according to standard operating procedures and that are triggered when certain conditions are exceeded.

**Critical Zone**: Typically the entire area where product and the containers and closures are exposed in aseptic processing.

**Detection Frequency**: The frequency with which contamination is observed in an environment. Typically expressed as a percentage of samples in which contamination is observed per unit of time.

**Environmental Isolates**: Microorganisms that have been isolated from the environmental monitoring program.

**Environmental Monitoring Program**: Documented program implemented via standard operating procedures that describes in detail the methods and acceptance criteria for monitoring particulates and microorganisms in controlled environments (air, surface, personnel gear). The program includes sampling sites, frequency of sampling, and investigative and corrective actions.

**Equipment Layout**: Graphical representation of an aseptic processing system that denotes the relationship between and among equipment and personnel. This layout is used in the Risk Assessment Analysis to determine sampling site and frequency of sampling based on potential for microbiological contamination of the product/container/closure system. Changes must be assessed by responsible managers, since unauthorized changes in the layout for equipment or personnel stations could result in increase in the potential for contamination of the product/container/closure system.

**Isolator for Aseptic Processing**: An aseptic isolator is an enclosure that is over-pressurized with HEPA filtered air and is decontaminated using an automated system. When operated as a closed system, it uses only decontaminated interfaces or rapid transfer ports (RTPs) for materials transfer. After decontamination they can be operated in an open manner with the ingress and/or egress of materials through defined openings that have been designed and validated to preclude the transfer of contamination. It can be used for aseptic processing activities or for aspesis and containment simultaneously.

**Media Flow**: The flow of material and personnel entering controlled environments should follow a specified and documented pathway that has been chosen to reduce or minimize the potential for microbial contamination of the product/container systems. Deviation from the prescribed flow could result in increase in the potential for microbial contamination. Material/personnel flow can be changed, but the consequences of the changes from a microbiological point of view should be assessed by responsible managers and must be authorized and documented.

**Media Fill**: Microbiological simulation of an aseptic process by the use of growth media processed in a manner similar to the processing of the product and with the same container/closure system being used.

**Media Growth Promotion**: Procedure that references Growth Promotion Test of Aerobes, Anaerobes, and Fungi in Sterility Tests (71) to demonstrate that media used in the microbiological environmental monitoring program, or in media-fill runs, are...
capable of supporting growth of indicator microorganisms and of environmental isolates from samples obtained through the monitoring program or their corresponding ATCC strains.

**Product Contact Areas:** Areas and surfaces in a controlled environment that are in direct contact with either products, containers, or closures and the microbiological status of which can result in potential microbial contamination of the product/container/closure system.

**Restricted Access Barrier System (RABS):** An enclosure that relies on HEPA filtered air over-spill to maintain separation between aseptically gowned personnel and the operating environment. It is subject to a high level of disinfection prior to use in aseptic process. It uses decontaminated (where necessary) interfaces or RTPs for materials transfer. It allows for the ingress and/or egress of materials through defined openings that have been designed and validated to preclude the transfer of contamination. If opened subsequent to decontamination, its performance capability is adversely impacted.

**Risk Assessment Analysis:** Analysis of the identification of contamination potentials in controlled environments that establish priorities in terms of severity and frequency and that will develop methods and procedures that will eliminate, reduce, minimize, or mitigate their potential for microbial contamination of the product/container/closure system.

**Sampling Plan:** A documented plan that describes the procedures and methods for sampling a controlled environment; identifies the sampling sites, the sampling frequency, and number of samples; and describes the method of analysis and how to interpret the results.

**Sampling Sites:** Documented geographical location, within a controlled environment, where sampling for microbiological evaluation is taken. In general, sampling sites are selected because of their potential for product/container–closure contacts.

**Standard Operating Procedures:** Written procedures describing operations, testing, sampling, interpretation of results, and corrective actions that relate to the operations that are taking place in a controlled environment and auxiliary environments. Deviations from standard operating procedures should be noted and approved by responsible managers.

**Sterile or Aseptic Field:** In aseptic processing or in other controlled environments, it is the space at the level of or above open product containers, closures, or product itself, where the potential for microbial contamination is highest.

**Sterility:** Within the strictest definition of sterility, an article is deemed sterile when there is complete absence of viable microorganisms. *Viable,* for organisms, is defined as having the capacity to reproduce. Absolute sterility cannot be practically demonstrated because it is technically unfeasible to prove a negative absolute. Also, absolute sterility cannot be practically demonstrated without testing every article in a batch. Sterility is defined in probabilistic terms, where the likelihood of a contaminated article is acceptably remote.

**Swabs for Microbiological Sampling:** Devices used to remove microorganisms from irregular or regular surfaces for cultivation to identify the microbial population of the surface. A swab is generally composed of a stick with an absorbent tip that is moistened before sampling and is rubbed across a specified area of the sample surface. The swab is then rinsed in a sterile solution to suspend the microorganisms, and the solution is transferred to growth medium for cultivation of the microbial population.

**Trend Analysis:** Data from a routine microbial environmental monitoring program that can be related to time, shift, facility, etc. This information is periodically evaluated to establish the status or pattern of that program to ascertain whether it is under adequate control. A trend analysis is used to facilitate decision-making for requalification of a controlled environment or for maintenance and sanitization schedules.

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**APPENDIX**

**Additional Resources**


(1125) NUCLEIC ACID-BASED TECHNIQUES—GENERAL

SCOPE

Nucleic acid-based assays are used in a variety of settings, the most common of which include the detection of infectious agents (viruses, bacteria, etc.), and cellular materials, as well as disease profiling. More recently such assays have also been used for forensic purposes and for the detection of trace contamination in biological materials. The latter include pharmaceutical development applications, such as viral clearance and adventitious agent testing in vaccine seed lots and tissue culture cell banks. This chapter introduces a series of general information chapters that provide techniques that support procedures for the detection and analysis of nucleic acids (see Figure 1). The assays using these techniques may be presented in a USP general chapter or in a private specification.

Figure 1

The major requirement for any nucleic acid analytical procedure is the availability of pure, intact nucleic acids for analysis. The information in Nucleic Acid-Based Techniques—Extraction, Detection, and Sequencing (1126) discusses procedures available for nucleic acid extraction and handling. Hybridization is the core mechanism underlying many molecular biology techniques, and in addition to the detection of nucleic acids by absorbance and fluorescence measurements and size measurement by gel electrophoresis, this chapter also covers blotting and identification of nucleic acid species by hybridization assays using labeled probes. Hybridization probes are oligonucleotides that have a sequence that is complementary to the target of interest. Probes contain radioactive, fluorescent, biotin, digoxigenin, or other tags that, upon binding of the probe to the target, allow visualization and identification of the target. Probes are capable of detecting target sequences that are present in concentrations too low to be detected by absorbance measurements or gel electrophoresis.

These analytical procedures require a minimum quantity of nucleic acid, typically in the nanogram to microgram range. However, in the vast majority of cases, e.g., in the detection of viruses or rare cellular RNA species, the nucleic acid under assay is present in minute quantities (in the picogram to femtogram range), and an amplification step must be performed before the nucleic acid can be detected and identified. The amplification step may be directed either at the signal used for detection (signal amplification), such as the branched DNA assay (bDNA assay), or at the target as in nucleic acid amplification technologies (NAT).

In 1983 a revolutionary yet simple process termed polymerase chain reaction (PCR) was developed for amplifying the number of specific nucleic acid fragments present in a sample, and in just a few years after its discovery PCR became the most frequently used procedure for amplifying nucleic acids, especially DNA. Since the inception of PCR, the number of applications has
expanded rapidly, and the technique, which now includes quantitative and multiplex assays, is currently used in almost every field of research and development in biology and medicine. Numerous variations of assay procedures have been developed for specific analytes. The general information chapter, Nucleic Acid-Based Techniques—Amplification (1127), describes amplification procedures used for DNA and RNA analysis as well as qualitative and quantitative NAT assays. Signal amplification procedures in which the signals, typically fluorescent signals, are used to detect the nucleic acid of interest, are not very common. The major signal amplification procedure, the branched DNA or bDNA assay, is used predominantly for viral nucleic acid detection.

Quality assurance aspects of the methodology are also covered, together with a summary of current regulatory requirements for NAT assays. The need for globally comparable, accurate, and reliable results in the diagnostics field has driven the quest for, and development of, national and international standards within an increasingly sophisticated and metrologically sound, highly developed international regulatory environment devoted to the highest standards of regulatory science. Because NAT has become the most widely used of nucleic acid techniques, the majority of guidance documents and standards are related to NAT. The general information chapter, Nucleic Acid-Based Techniques—Microarrays (1128), addresses a still-emerging field that is of increasing relevance to molecular DNA analysis. Detailed treatment of various microarrays, including data analysis and validation, are excluded from (1128) at this time. The general information chapter, Nucleic Acid-Based Techniques—Genotyping (1129), focuses on the specific modifications of the techniques that are necessary to enable detection of single base differences and common genetic variations, e.g., single nucleotide polymorphisms (SNPs). The final general information chapter in the series, Nucleic Acid-Based Techniques—Approaches for Detecting Trace Nucleic Acids (Residual DNA Testing) (1130), describes residual DNA testing in the context of pharmaceutical manufacturing. Applications relevant to viral adventitious agents, however, are discussed in the general information chapter Virology Test Methods (1237).

Two major uses of nucleic acid testing are excluded from this family of NAT chapters: viral testing for blood and blood product safety and genetic testing. The traditional perspective of USP is to develop public standards that can be applied to a particular final product without expressly defining a product and/or its production details. This chapter aims to specify when traditional methodologies or existing standards can be adapted. Novel methodologies for amplification and detection by NAT are also highlighted. As these new methodologies become mature and properly validated, they will be included in subsequent revisions.

Due to rapid development in the field, compendial and regulatory affairs scientists are advised to consult the current edition of USP and its Supplements regularly.

**GLOSSARY**

**3′–5′ Exonuclease activity:** Enzymatic activity to remove a mispaired nucleotide from the 3′ end of the growing strand. The reaction is a hydrolysis of a phosphoester bond. The presence of a 3′–5′ exonuclease, or proofreading, activity improves the fidelity of the polymerization.

**5′–3′ Exonuclease activity:** Enzymatic activity to remove a mispaired nucleotide from the 5′ end of a polynucleotide strand. This activity is actually that of a single-strand-dependent endonuclease and is needed to remove RNA primers of Okazaki fragments, the RNA strand in the intermediate DNA–RNA heteroduplex during reverse transcription, and during DNA repair.

**Absorbance:** [Symbol: A] The logarithm, to the base 10, of the reciprocal of the transmittance (T). [NOTE—Descriptive terms used formerly include optical density, absorbancy, and extinction.]

**Accuracy:** The accuracy of an analytical procedure is the closeness of test results obtained by that procedure to the true value.

**Allele:** One of two or more alternative forms of a gene at a given position (locus) on a chromosome, caused by a difference in the sequence of DNA.

**Amplimer:** A short segment of DNA generated by the PCR process whose sequence is defined by forward and reverse primers. Sometimes referred to as an amplier.

**Amplification:** The enzymatic in vitro replication of a target nucleic acid.

**Annealing:** Hybridizing or binding of complementary nucleic acids, usually at an optimal temperature.

**Concatenation:** The process in which a DNA segment composed of repeated sequences is linked end-to-end.

**Complementary dna (cdna):** DNA synthesized from an RNA template in an enzymatic reaction catalyzed by the enzyme reverse transcriptase.

**Denaturation:** The process of separating double-stranded DNA into single strands by breaking the hydrogen bonds. This is typically accomplished by heating the DNA solution to temperatures greater than 90° or by treating it with strong alkali.

**Deoxyribonucleic acid (dna):** The genetic material that is passed from parent to daughter cells and propagates the characteristics of the species in the form of genes it contains and the proteins for which it codes. DNA contains the following four deoxyribonucleosides: dA, dC, dT, and dG.

**Deoxyribonucleotide triphosphate (dntp):** A base that is added to a primer during the PCR that comprises the newly synthesized strand. Examples of dNTPs are dATP, dUTP, dCTP, dGTP, and dTTP.

**Detection limit:** It is the lowest amount of analyte in a sample that can be detected, but not necessarily quantitated, under the stated experimental conditions.

**DNA polymerase:** An enzyme that can synthesize new complementary DNA strands using a DNA template and primer. Several of these enzymes are commercially available, including Taq DNA polymerase and rTth DNA polymerase.

**Energy transfer:** This describes the process in which an excited state of one molecular entity (the donor) is deactivated to a lower-lying state by transferring energy to a second molecular entity (the acceptor), which is thereby raised to a higher energy state.

**Extension:** Refers to the elongation of the DNA chain that is being synthesized using the parent DNA strand as the template for synthesis of that daughter strand. This is a natural process that occurs during DNA replication. Extension occurs during the PCR process with DNA polymerases.

**Extinction coefficient:** [Symbol: ε]—The quotient of the absorbance (A) divided by the product of the concentration, expressed in moles/L, of the substance and the absorption path length, in cm. [NOTE—Terms formerly used include molar absorbancy index; molar absorptivity; and molar absorption coefficient.]
Fidelity: Fidelity is a measure of the accuracy of nucleic acid replication. The polymerase enzyme used is only one of the elements that influences fidelity. Other elements include buffer conditions, thermal cycling parameters, number of cycles, efficiency of amplification, and the sequence of the DNA being copied.

Fluorophore: A functional group in a molecule that makes the molecule fluorescent by absorbing energy of a specific wavelength and re-emits the energy at another wavelength.

Fluorescence: The emission of one or more photons by a molecule or atom activated by the absorption of a quantum of electromagnetic radiation. X-rays, UV, visible light, and IR radiations may all stimulate fluorescence. For details on the spectroscopic measurement of fluorescence, see Fluorescence Spectroscopy (853).

Genome: The complete genetic complement or the complete set of instructions for reproducing an organism and carrying out its biological function in life. The DNA in our cells comprises our genome. When our cells divide, the complete genome in our cells is duplicated for transmission to each of the remaining daughter cells.

Genotype: The genetic constitution of an organism as revealed by genetic or molecular analysis, i.e., the complete set of genes, both dominant and recessive, possessed by a particular cell or organism.

Genotyping: The process of assessing genetic variations present in an individual.

Hairpin: Antiparallel duplex structure that forms by pairing of inverted repeat sequences within a single-stranded nucleic acid. The helical section is called the stem and the unpaired base segment at the end of the structure is called the loop.

Hot-start PCR: Technique that is commonly used to improve the sensitivity and specificity of PCR amplification. A hot start is performed by withholding from the reaction mix a key component necessary for amplification until the reaction reaches a temperature above the optimal annealing temperature of the primers. The component withheld from the reaction mix can be primers, DNA polymerase, MgCl₂, or dNTPs.

Hybridization: The process of forming a double-stranded nucleic acid molecule, for example between a nucleotide sequence (probe) and a target.

Ligation: The process of joining two or more DNA fragments.

Melting temperature (Tm): The temperature at which 50% of the DNA becomes single-stranded.

Microarray: Sets of miniaturized chemical reaction areas that are used to test DNA fragments, antibodies, or proteins. Usually the probes are immobilized on a chip and hybridized with target.

Mismatch: Sets of conventional base pairing (other than C with G, and A with T or U). A mismatched base pair has lower bonding energy and decreases the stability of the DNA molecule.

Nucleic acid: Linear polymers of nucleotides, linked by 3', 5' phosphodiester linkages. In DNA, deoxyribonucleic acid, the sugar group is deoxyribose, and the bases consist of adenine, guanine, thymine, and cytosine. RNA, ribonucleic acid, has ribose as the sugar, and uracil replaces thymine.

Oligonucleotide: Linear sequence comprising as many as 25 nucleotides joined by phosphodiester bonds, generally used as a DNA synthesis primer.

Photobleaching: Photobleaching is the irreversible destruction of a fluorophore in the excited state. Different fluorophores have different rates of photobleaching. For example, fluorescein photobleaches very easily. Often the rate of decomposition is proportional to the intensity of illumination. A simple and practical way to overcome this is to reduce the incident radiation.

Polymerase: An enzyme that catalyzes the synthesis of nucleic acids on pre-existing nucleic acid templates, assembling RNA from ribonucleotides or DNA from deoxyribonucleotides.

Polymerase chain reaction (PCR): A laboratory technique that rapidly amplifies a specific region of double-stranded DNA, predetermined by the pair of primers used for amplification. Generally involves the use of a heat-stable DNA polymerase.

Precision: The degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of a homogenous sample.

Primer: Nucleic acid polymers link a mononucleotide to a chain of nucleic acids, which is called the primer. RNA polymerases are able to use a single nucleotide as a primer, but DNA polymerases always require an oligonucleotide.

Probe: A specific DNA or RNA sequence that has been labeled by radioactive, fluorescent, or chemiluminescent tags and is used to detect complementary sequences by hybridization techniques such as blotting or colony hybridization. In addition, probes can also be used for quantitation of amplicons as described for quantitative PCR assays. A more detailed description of such probes is given in the general information chapter, Nucleic Acid-Based Techniques—Amplification (1127).

Processivity: The ability of an enzyme to repetitively continue its catalytic function without dissociating from its substrate.

Proofreading activity: Literally to read for the purpose of detecting errors for later correction. DNA polymerase has a 3' to 5' exonuclease activity that is used during polymerization to remove recently added nucleotides that are incorrectly paired.

Quantitation limit: It is the lowest amount of analyte in a sample that can be determined with an acceptable precision and accuracy under the stated experimental conditions.

Quenching: The process of extinguishing, removing, or diminishing a physical property such as heat or light. Fluorescence quenching can be either collisional or static.

Reverse transcriptase: An enzyme that requires a DNA primer and catalyzes the synthesis of a DNA strand from an RNA template. An enzyme that can use RNA as a template to synthesize DNA.

Reverse transcription (rt): The process of making cDNA using an RNA template.

Real-time PCR: May often be referred to as Quantitative PCR or Real-Time Quantitative PCR but not RT-PCR and is a procedure for simultaneous DNA quantitation and amplification. The generation of amplicons monitored as they are generated by the use of a fluorescent reporter system and captured by sophisticated instrumentation.

Real-time (RT-PCR): The combination of real-time PCR and reverse transcription PCR.

Reverse transcriptase polymerase chain reaction (RT-PCR): A variation of the PCR technique in which cDNA is made from RNA via reverse transcription. The cDNA is then amplified using standard PCR protocols.

Ribonucleic acid (RNA): A type of nucleic acid composed of a specific sequence of ribonucleotides linked together. RNA contains the following four ribonucleosides: A, C, G, and U.

Robustness: The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in procedural parameters listed in the procedure documentation and provides an indication of its suitability during normal usage.

Not official text. Please refer to the currently official version of the applicable USP–NF or FCC standard for compliance purposes.

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**rTth dna polymerase**: Recombinant thermostable DNA polymerase originally isolated from the bacterium *Thermus thermophilus*. rTth has optimal activity at 70°–80° and survives the denaturation steps of PCR. In addition to DNA polymerase activity, it has efficient reverse transcriptase activity in the presence of manganese.

**Specificity**: The ability to assess unequivocally the analyte in the presence of components that may be expected to be present, such as impurities, degradation products, and matrix components.

**Taq dna**: Thermostable DNA polymerase that is originally isolated from the bacterium *Thermus aquaticus*, Taq has optimal activity at 70°–80° and is not degraded during the high-heat denaturation steps of PCR.

**Template**: A master copy used to start the DNA or RNA replication process.

**Transcription**: The synthesis of RNA using a DNA template.

### APPENDICES

#### Appendix 1: Regulations and Standards

Nucleic acid-based techniques have rapidly transformed almost every field of research, pharmaceutical development, and diagnostics. The need for globally comparable, accurate, and reliable results in the diagnostic field has driven the development of national and international standards as well as fostered a highly developed regulatory environment. Because NAT has become the most widely used of nucleic acid techniques, the majority of guidance documents and standards are related to NAT.\(^1\) Virus-specific regulations and reference standards will be addressed in the Appendix to General Information chapter *Virology Test Methods* (1237). The following is a selective list of national guidance documents. For application-specific guidance the compendial user is referred back to the relevant regulatory agency for the most current guidance.

- FDA Center for Biologics Evaluation (CBER) “Nucleic Acid Based In Vitro Diagnostic Devices for Detection of Microbial Pathogens” (2005)
- FDA Center for Biologics Evaluation (CBER) “Guidance for Industry: Content and Format of Chemistry, Manufacturing and Controls Information and Establishment Description Information for a Biological In Vitro Diagnostic Product” (1999)

#### Appendix 2: Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>AABB</td>
<td>American Association of Blood Banks</td>
</tr>
<tr>
<td>ACD</td>
<td>acid citrate dextrose</td>
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<tr>
<td>ASO</td>
<td>allele-specific oligonucleotides</td>
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<tr>
<td>bDNA</td>
<td>branched DNA assay</td>
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<tr>
<td>BMA</td>
<td>bone marrow aspirate</td>
</tr>
<tr>
<td>CE-LIF</td>
<td>capillary electrophoresis and laser-induced fluorescence</td>
</tr>
<tr>
<td>CCD</td>
<td>charge-coupled device</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CPR</td>
<td>cyclic probe reaction</td>
</tr>
<tr>
<td>C</td>
<td>Cl, cycle threshold</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethylpyrocarbonate</td>
</tr>
<tr>
<td>DHPLC</td>
<td>denaturing high-performance liquid chromatography</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNase</td>
<td>deoxyribonuclease</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxyribonucleotide triphosphate</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>dNTP</td>
<td>dinucleotide triphosphate</td>
</tr>
<tr>
<td>DOP-PCR</td>
<td>degenerated oligonucleotide primed PCR</td>
</tr>
<tr>
<td>dsDNA</td>
<td>double-stranded DNA</td>
</tr>
<tr>
<td>ssDNA</td>
<td>single-stranded DNA</td>
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</tbody>
</table>

\(^1\) Reference materials for nucleic acid-based techniques are available from National Institute of Standards and Technology (NIST), http://ts.nist.gov/measurementservices/referencematerials/index.cfm.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>dUTP</td>
<td>2′-deoxyuridine 5′-triphosphate</td>
</tr>
<tr>
<td>dTTP</td>
<td>2′-deoxythymidine 5′-triphosphate</td>
</tr>
<tr>
<td>ESI</td>
<td>electrospray ionization</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FEN</td>
<td>flap endonuclease</td>
</tr>
<tr>
<td>FISH</td>
<td>fluorescent in situ hybridization</td>
</tr>
<tr>
<td>FFPE</td>
<td>formalin-fixed paraffin embedded</td>
</tr>
<tr>
<td>FRET</td>
<td>fluorescence resonance energy transfer</td>
</tr>
<tr>
<td>GLP</td>
<td>good laboratory practice</td>
</tr>
<tr>
<td>HCV</td>
<td>hepatitis C virus</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>ICH</td>
<td>International Conference on Harmonization</td>
</tr>
<tr>
<td>LAPS</td>
<td>light-addressable potentiometric sensor</td>
</tr>
<tr>
<td>LCR</td>
<td>ligase chain reaction</td>
</tr>
<tr>
<td>LED</td>
<td>light-emitting diode</td>
</tr>
<tr>
<td>LNA</td>
<td>locked nucleic acid</td>
</tr>
<tr>
<td>MALDI</td>
<td>matrix-assisted laser desorption–ionization</td>
</tr>
<tr>
<td>MDA</td>
<td>multiple-displacement amplification</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-([N\text{-morpholino}]\text{propanesulfonic acid})</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>NAT</td>
<td>nucleic acid amplification technologies</td>
</tr>
<tr>
<td>NASBA</td>
<td>nucleic acid sequence-based amplification</td>
</tr>
<tr>
<td>NTP</td>
<td>nucleotide triphosphate</td>
</tr>
<tr>
<td>OLA</td>
<td>oligonucleotide ligation assay</td>
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<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PEP</td>
<td>primer-extension-preamplification</td>
</tr>
<tr>
<td>PPI</td>
<td>pyrophosphate</td>
</tr>
<tr>
<td>QA</td>
<td>quality assurance</td>
</tr>
<tr>
<td>QC</td>
<td>quality control</td>
</tr>
<tr>
<td>RCA</td>
<td>rolling circle amplification</td>
</tr>
<tr>
<td>RFLP</td>
<td>restriction fragment length polymorphism</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNAse</td>
<td>ribonuclease</td>
</tr>
<tr>
<td>RT</td>
<td>reverse transcriptase</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcription-polymerase chain reaction</td>
</tr>
<tr>
<td>rTth</td>
<td>recombinant <em>Thermus thermophilus</em></td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
</tr>
<tr>
<td>SSR</td>
<td>self-sustained sequence replication</td>
</tr>
<tr>
<td>SSCP</td>
<td>single-strand conformation polymorphism</td>
</tr>
<tr>
<td>STR</td>
<td>short tandem repeat</td>
</tr>
<tr>
<td>Taq</td>
<td><em>Thermus aquaticus</em></td>
</tr>
<tr>
<td>Tm</td>
<td>melting temperature; the temperature at which 50% of the double-stranded nucleic acid molecule becomes single-stranded</td>
</tr>
<tr>
<td>TMA</td>
<td>transcription-mediated amplification</td>
</tr>
</tbody>
</table>
NUCLEIC ACID EXTRACTION

Introduction

The basic principles of nucleic acid amplification technology (NAT) and definitions of the various techniques are covered in Nucleic Acid-Based Techniques—General (1125). The current chapter covers general steps in the extraction and purification of nucleic acids from a variety of samples.

The expanding discipline of molecular biology in pharmaceutical and biomedical research and development is characterized by the rapid discovery of new markers for disease and technologies for their detection. Nucleic acid targets are isolated from a wide variety of specimens, and the quality and quantity of the extracted target are highly affected by specimen collection, handling, and choice of extraction procedure.

The analysis of complex organisms by molecular biological techniques requires the isolation of pure, high molecular weight genomic DNA and intact full-length RNA. The application of these techniques then allows the detection, identification, and characterization of the associated organism or adventitious agent. Recently developed tests employing purified human DNA enable genetic testing for the presence, predisposition, or carrier status of inherited diseases such as cystic fibrosis, hereditary hemochromatosis, or Tay–Sachs disease, to name a few examples, or the analysis of single nucleotide polymorphisms (SNPs).

DNase and RNase are the major sources of nucleic acid instability. Although both enzymes are ubiquitous and are easily released during nucleic acid extraction, RNases are far more stable and harder to inactivate than are DNases because they generally do not require co-factors in order to function. Minute amounts of RNase are sufficient to destroy RNA, so great care should be taken to avoid inadvertently introducing these enzymes into the sample during or after the isolation procedure. If RNA is collected for the specific application of gene expression analysis, researchers should keep in mind that the sample collection process itself can alter the resulting expression profile.

Because of the ubiquity of RNases, measurement of intracellular RNA targets has lagged behind that of DNA targets in contributing to patient management and characterization of targets for pharmaceutical purposes. However, RNA represents the current status of the organism and is an important tool for correlating a phenotype with its associated genetic activity. The unstable nature of RNA has made standardization of NAT tests difficult, and false negative results can easily arise from a poorly handled sample because of target degradation rather than from the absence of disease or regulation of gene activity.

Nevertheless, commercially available isolation and detection systems provide a high level of standardization and robustness, resulting in the implementation of RNA-based assays in recent years. The following sections discuss general steps in the extraction and purification of nucleic acids from a variety of samples, focusing on (1) collection, handling and storage of samples; (2) disruption of samples; (3) subsequent extraction and purification of nucleic acids; and (4) storage of purified nucleic acids.

Sample Source

The broad diversity of possible specimens requires different procedures for collection. For example, blood samples are collected in an appropriate anticoagulant- or additive-containing tube. Ethylenediaminetetraacetic acid (EDTA) and acid citrate dextrose (ACD) are the recommended anticoagulants for tests that require plasma or bone marrow aspirate (BMA) samples. When extraction from tissues is appropriate, the optimal amount of tissue is usually 1 to 2 g, depending on the type of tissue, because the amount of DNA and RNA per weight of tissue varies greatly from tissue to tissue. In general, more than 10 mg of tissue is required to obtain >10 µg of DNA or RNA. Because of the highly variable amounts and types of proteins and other contaminants present in different tissues, nucleic acid isolation protocols are tissue-specific, and a broad range of ready-to-use isolation systems are available from different manufacturers of kits for nucleic acid extraction. The tissue type also influences the stability of both DNA and RNA in specimens, and the two types of nucleic acid differ importantly with respect to sample preparation and downstream analysis. These issues are described later in the chapter.

Pre-Analytical Steps and Sample Collection

Although the genetic makeup of the organism remains mostly unchanged over time, the mRNA population represents the current status of a cell under any given set of conditions, and thus is highly dynamic. To prevent degradation of mRNA and/or to preserve the original transcription pattern of the cellular mRNA, tissue should be placed immediately on ice or snap-frozen in liquid nitrogen. However, freezing disrupts the cellular structure and releases RNases. Hence, for RNA isolation in general (mRNA, ribosomal RNA, viral RNA, etc.), thawing in an RNase-inactivating buffer is essential. A more convenient procedure employs a stabilizing agent at ambient temperature. Several reagents for different types of sample material (e.g., tissue or bacteria) are commercially available. Vanadium salts were once used to inhibit RNase activity, but they have been superseded.
by the use of chaotropic agents for the inhibition of RNase and stabilization of RNA. The sample can easily be collected in such reagents and stored for several days to weeks prior to RNA isolation.

For reliable gene-expression analysis, the immediate stabilization of the RNA expression pattern and of the RNA itself is an absolute prerequisite. Directly after the biological sample is harvested or extracted, changes in the gene-expression pattern occur because of specific and nonspecific RNA degradation as well as transcriptional induction. Such changes in the gene-expression pattern should be avoided for all reliable quantitative gene-expression analyses, such as biochip and array analyses and quantitative reverse transcription-polymerase chain reaction (RT-PCR).

The use of gloves while handling reagents and RNA samples is mandatory to prevent RNase contamination arising from contact with the surface of the skin or from laboratory equipment. In order to create and maintain an RNase-free environment, laboratory personnel should treat water or buffer solutions with diethylpyrocarbonate (DEPC), which inactivates RNases by covalent chemical modification. Care should be taken because DEPC is irritating to the eyes, skin, and mucous membranes and is also a suspected carcinogen. Alternatively, commercially available RNase-free solutions and reagents may be used. Commercially available RNase inhibitor proteins are also available for use in reactions but with different levels of effectiveness with respect to various RNase types. However, it should be noted that DEPC cannot be used with Tris-buffered solutions. Many scientists recommend the use of disposable vessels when working with RNA. Nondisposable glassware should be cleaned with a detergent, thoroughly rinsed, and oven baked at 240°C for 4 or more hours before use (autoclaving alone will not fully inactivate many RNases). Alternatively, glassware can also be treated with DEPC. Nondisposable plasticware should be thoroughly rinsed with 0.1 M sodium hydroxide and 1 mM EDTA, followed by RNase-free water. Alternatively, chloroform-resistant plasticware can be rinsed with chloroform to inactivate RNases. The use of aerosol-resistant filter tips is also important for avoiding RNase contamination. These issues are not critical for DNA, and following the rules of Good Laboratory Practice (GLP) is generally sufficient for successful isolation of DNA.

As a general precaution, staff should follow all applicable safety precautions when handling tissue or body fluids (human or other). Some of these precautions (e.g., the use of disposable gloves) also prevent contamination of the sample. Applicable guidelines and standards for the collection and processing of human-derived materials have been published by the American Association of Blood Banks, the International Conference on Harmonization, and the FDA.

Sample Disruption and Homogenization

Prior to extraction, source material is disrupted and homogenized. Disruption is the complete breakage of cell walls and plasma membranes of solid tissues and cells in order to release all DNA and RNA contained in the specimen. This is usually done using a lysis buffer that also inactivates endogenous nucleases. In addition to disrupting tissues, homogenization shears high molecular weight DNA and cellular components. During RNA isolation, scientists often must reduce the viscosity of cell lysates (caused by the presence of high molecular weight DNA) prior to final isolation and analysis. In general, the same procedures are applicable for extraction of DNA and RNA. For DNA isolation more gentle procedures are preferable, but during RNA isolation, cells and tissues can be disrupted using a mixer mill because there is no risk of shearing the RNA. Certain downstream applications require high molecular weight DNA, and care should be taken not to shear the DNA molecules and thus render the DNA unsuitable for further analysis.

Extraction and Purification

Although several procedures are available for nucleic acid extraction, the suitability of a procedure depends on the starting material, the type and purity of nucleic acid isolated, and possibly the downstream application. The principal procedures are described below; several commercial kits are available to accommodate different sample types and applications.
PHASE EXTRACTION

The original technique for extraction of DNA and RNA from lysed samples is phase extraction, which involves nucleic acid extraction using a mixture of phenol and chloroform. Depending on pH and salt concentration, either DNA or RNA partitions in the aqueous phase. At neutral/basic pH, the DNA remains in the aqueous phase, and RNA remains in the organic phase or in the interphase (with the proteins). However, at acidic pH, DNA in the sample is protonated, neutralizing the charge and causing it to partition into the organic phase. RNA, which remains charged, partitions in the aqueous phase. The two phases are separated by centrifugation, and the aqueous phase is re-extracted with a mixture of phenol and chloroform, followed by extraction with chloroform to remove any residual phenol. The nucleic acid is recovered from the aqueous phase by precipitation with alcohol. For RNA, this procedure is often combined with a protease digestion, alcohol or lithium chloride precipitation, and/or cesium chloride (CsCl) density gradients. A potential problem is contamination of the recovered DNA or RNA with organic solvents that may interfere with enzymatic downstream applications or spectrometry readouts.

CESIUM CHLORIDE DENSITY GRADIENT CENTRIFUGATION

For the isolation of high molecular weight genomic DNA, CsCl density gradient centrifugation is the traditional procedure. Cells are lysed using a detergent, and the DNA is isolated from the lyse by alcohol precipitation. The DNA is then mixed with CsCl and ethidium bromide and centrifuged for several hours at a high g force (typically 100,000 × g). The DNA band, which can be visualized under UV light as a result of the intercalation of the ethidium bromide with the DNA, is collected from the centrifuge tube, extracted with isopropanol to remove the ethidium bromide, and then precipitated with ethanol to recover the DNA. This procedure allows the isolation of high-quality DNA, but it is time consuming and also a safety concern because of the high quantity of EtBr involved.

ANION-EXCHANGE CHROMATOGRAPHY

An alternative procedure for the purification of high molecular weight genomic DNA is anion-exchange chromatography based on the interaction between the negatively charged phosphate groups of the nucleic acid and positively charged surface molecules on the anion-exchange resin. Binding occurs under low-salt conditions, and impurities such as RNA, cellular proteins, and metabolites are washed away using medium-salt buffers. Pure DNA is eluted with a high-salt buffer and is desalted and concentrated by alcohol precipitation. This procedure yields DNA of a purity and biological activity equivalent to two rounds of purification in CsCl gradients, but in much less time. The procedure also avoids the use of toxic substances, and it can be adapted for different scales of purification. DNA up to 150 kilobases (kb) in length may be isolated using this procedure. Several kits are available for the isolation of DNA based on anion-exchange technology, and procedures vary in processing times and the quality and size of the isolated DNA.

SILICA TECHNOLOGY

The current procedure of choice for most applications is based on silica technology and can be used for isolation of full-length RNA or DNA with an average size of 20 to 50 kb. However, higher molecular weight DNA exceeding 100 kb is not efficiently extracted by this technology. The procedure relies on the selective adsorption of nucleic acids to silica in the presence of high concentrations of chaotropic salts. Although both types of nucleic acid adsorb to silica, the use of specific buffers in the lysis procedure ensures that only the desired nucleic acid is adsorbed while other nucleic acids, cellular proteins, and metabolites remain in solution. The contaminants are washed away, and high-quality RNA or DNA is eluted from the silica using a low-salt buffer. The silica matrix can be used as particles in suspension, in the form of magnetic beads, or as a membrane. This technique is suitable for high throughput, and several kits and automated systems are commercially available. However, these aqueous lysis buffers (in contrast to lysis buffers based on an organic solvent such as phenol) are not ideally suited for difficult-to-lyse samples (e.g., fatty tissues). Kits designed to facilitate lysis of fatty tissues and to inhibit RNases are available. Silica-based kits provide a fast and reliable procedure for both DNA and RNA purification and are commonly used for nucleic acid extraction. Although these procedures yield pure nucleic acids, for some applications in which even trace contaminations with either RNA or DNA may interfere, pretreatment with DNase or RNase may be necessary. Alternatively, procedures that use specific probe capture may be used. Relevant applications requiring such ultra-pure nucleic acids are discussed in Nucleic Acid-Based Techniques—Amplification (1127).

Specific Applications for Hard-to-Extract Materials

EXTRACTION FROM FORMALIN-FIXED AND PARAFFIN-EMBEDDED BIOPSIES

The nucleic acids in formalin-fixed paraffin embedded (FFPE) biopsies are usually heavily fragmented and chemically modified by formaldehyde. Although formaldehyde modification cannot be detected in standard quality control assays such as gel electrophoresis, formaldehyde modification does interfere with enzymatic analyses. Sufficient extraction and demodification for DNA can be achieved by prolonged digestion with protease, but this will lead to heavy fragmentation and degradation of RNA. Some isolation systems have been optimized to reverse as much formaldehyde modification as possible without further RNA degradation. Nevertheless, RNA purified from FFPE samples should not be used in downstream applications that require full-length RNA. Some applications may require modifications to allow the use of fragmented RNA (e.g., designing small amplicons for RT-PCR).
EXTRACTION FROM BACTERIA AND PATHOGENS

Although Gram-negative bacteria are relatively easy to lyse, Gram-positive bacteria or yeasts typically need an enzymatic pretreatment to remove the cell wall for efficient lysis. This methodology can be applied only to DNA isolation because the enzymatic treatment will influence the expression profile of the organism, and therefore RNA isolation requires a more rapid lysis procedure. Another factor to consider is that microorganisms normally occur against the background of a host or an environmental matrix (e.g., soil), which makes detection by polymerase chain reaction (PCR) often difficult because of inhibitory components. This means that the isolation procedure has to be carefully adapted and optimized for the specific organism and sample type. Commercial kits are available, and most are based on the use of lysozyme for the removal of cell walls.

SPECIAL CONSIDERATIONS FOR LIMITED SAMPLE AMOUNTS

Multiple genetic testing techniques, including SNP analysis, short tandem repeat analysis, sequencing or genotyping using arrays, real-time PCR, and other procedures depend on the availability of high-quality DNA. Because human genomic DNA or samples of individual genotypes are often limited, a process to immortalize nucleic acid samples can overcome this limitation. Procedures applicable to genotyping are discussed in *Nucleic Acid-Based Techniques—Genotyping* (1129). Whole-genome amplification (WGA) has recently been employed to amplify limited genomic DNA from already purified DNA or directly from clinical or casework samples without any DNA purification. Two basic technologies for WGA are available and are PCR-based or rely on isothermal multiple-displacement amplification. These applications are described in more detail in *Nucleic Acid-Based Techniques—Amplification* (1127).

Sample Handling and Long-Term Storage

DNA is a relatively stable macromolecule, and once isolated it can be kept at 2° to 8° for at least 1 year. However, where DNA is present in very small quantities, such as in a test of residual DNA, it may be advisable to store the DNA at less than or equal to −20°. Generally, DNA is stored in solution. Distilled water can be used if DNA will be used for PCR and/or endonuclease digestion within a few days after its isolation. However, Tris–EDTA at pH 7.5–8.5 is the preferred buffer for DNA storage because DNA degradation can occur in water because of the limited buffering capacity of this medium. Purified nucleic acids retain recognizable characteristics during long-term storage, provided the samples are stored as frozen solutions. The DNA solution should be stored as a primary stock solution frozen at −80°. DNA can also be lyophilized and stored dry without the need for refrigeration. In some cases DNA can be stored for years on special filter papers that bind DNA and allow storage in a dried state at ambient temperature.

The ubiquity of RNases requires extra precautions when handling RNA. Isolated RNA should be kept on ice when aliquots are pipetted. Filter tips that prevent RNase carry-over from the pipette and sterile, disposable polypropylene tubes are recommended throughout the procedure because these tubes are generally RNase-free and do not require any pretreatment to inactivate RNases. Purified RNA can be stored at −20° or −80° in water. Under these conditions no degradation is normally detectable. Unlike DNA, RNA does not benefit from basic buffer solutions during long-term storage because of its sensitivity to alkaline conditions. Generally, if nucleic acid samples are required for multiple testing, RNA and DNA samples should be frozen in multiple aliquots at −80° for subsequent analysis in order to avoid repeated freeze–thaw cycles that can lead to degradation, and also to minimize the possibility of contamination, which could result in analytical inaccuracy.

QUALITATIVE AND QUANTITATIVE EVALUATION OF NUCLEIC ACIDS

Introduction

This section describes procedures that assess the purity, integrity, and quantity of purified nucleic acids, including spectroscopic procedures, electrophoresis of nucleic acid fragments, and probe-based techniques. Detection and quantitation by amplification are discussed in *Nucleic Acid-Based Techniques—Amplification* (1127).

ABSORBANCE SPECTROSCOPY

The basic principles of spectroscopy are addressed in *Ultraviolet-Visible Spectroscopy* (857). For nucleic acids, absorbance is determined at 260 nm, but this procedure does not distinguish between DNA and RNA. Absorbance can also be used to estimate protein contamination in nucleic acids. Proteins maximally absorb at 280 nm, and nucleic acids maximally absorb at 260 nm. Thus the calculation of the A260/A280 ratio is used as an estimation of protein contamination in nucleic acid preparations. A ratio of 1.8 to 2.0 is considered desirable. As an example, double-stranded DNA has an extinction coefficient of 20 for 1 mg per mL of DNA at 260 nm and a coefficient of 10 at 280 nm. In contrast, for 1 mg per mL of protein, the extinction coefficients are on the order of 1 at 280 nm (depending on tyrosine and tryptophan content) and 0.57 at 260 nm. Thus a large protein contamination could exist at a 260/280 ratio of greater than 1.8 because of the lower sensitivity of protein absorbance. In addition, the change of absorbance of DNA with wavelength (ΔA/Δλ) is steep at 280 nm, and this could lead to an incorrect determination if the spectrophotometer is out of calibration. The peak at 260 nm is broad, and thus readings are less sensitive to calibration issues.

Information on contamination by nonproteinaceous materials can be provided by a scan of DNA from 220 nm to 320 nm. Pure DNA has a mostly symmetric peak around 260 nm, zero absorbance at 320 nm, and a minimum at 230 nm. Absorbance rises again from 230 nm to 220 nm. Interfering substances can co-purify with DNA and absorb in the lower UV range (around 230 nm). These substances can interfere with and lead to an overestimation of DNA content, thus showing the utility of a scan—or at least a measurement of absorbance—at 230 nm in addition to 260 nm and 280 nm. Absorbance above 300 nm can arise from other contaminants and particulate matter. Common reagents used in the isolation of DNA, particularly solvents
such as phenol and alcohols if they are not completely removed, can interfere with DNA absorbance measurements. Analysts should be aware of the limitations of this type of measurement. Finally, the absorbance of DNA and the 260/280 ratio is dependent on ionic strength—a difference as large as 30% can exist. Absorbance of genomic DNA is higher, and the 260/280 ratio is lower in pure water when compared with the same DNA in a buffer or a salt solution.

For the purposes of quantitation of nucleic acids, the respective extinction coefficients for DNA and RNA are used. An absorbance of 1 in a 1-cm cuvette corresponds to 50 µg per mL of double-stranded DNA \( [E (\text{specific absorption coefficient}) = 0.02 (\mu g \text{ per mL}^{-1} \text{ cm}^{-1})] \). The specific absorption coefficient for RNA at 260 nm is \( E = 0.025 (\mu g \text{ per mL}^{-1} \text{ cm}^{-1}) \) (absorbance of 1.0 corresponds to 40 µg per mL), and for single-stranded DNA \( E = 0.027 \) (absorbance of 1.0 corresponds to 37 µg per mL). A solution of DNA is read against a blank of the same buffer solution in which the DNA is dissolved. Ideally, readings should fall within a range of 0.1 to 1.0 absorbance for adequate linearity. Absorbance above 1.0 becomes increasingly nonlinear as the absorbance rises. The accuracy of readings below 0.1 (5 µg per mL DNA) depends on the quality and noise level of the spectrophotometer.

**Fluorescence Protocols for DNA and RNA Quantitation**

Cyanine dye derivatives are used for the quantitation of nucleic acids because they specifically interact with nucleic acids (DNA, RNA, and oligonucleotides) and fluoresce only upon binding. The exact mechanism of interaction is not always fully understood but may involve intercalation in double-stranded DNA and surface binding.

Measurements can be performed using a fluorometer or a plate reader. The sensitivity of fluorescence with these dyes is much higher than that of absorbance, which gives these dyes great utility when DNA concentration is low (down to 25 pg per mL). The dye must be protected from light to avoid photobleaching. Linearity is maintained over three to four orders of magnitude. Calf thymus and Lambda phage DNA are often used as calibrants to construct a standard curve. Some of these dyes have been optimized to bind double-stranded DNA or single-stranded RNA and oligonucleotides. A DNA-binding dye will also bind to single-stranded DNA and RNA but at a lower ionic strength, and the signal is about 10% or less than that seen with double-stranded DNA for an equivalent mass of material. Thus, this methodology is preferred for DNA measurements when no effort has been made to remove RNA from the preparation. Another fluorescent dye is available and is optimized for RNA measurements. Using two different concentrations of this dye, analysts can detect RNA in amounts as low as 1 ng per mL and as high as 1 µg per mL. The dye also fluoresces with DNA but does not display an equivalent ability to minimize binding by the use of particular conditions (e.g., with DNA and the double-strand binding dye). Quantitation may be affected by contaminating nucleic acid (e.g., DNA in an RNA preparation and vice versa). Treatment with a DNase is needed if DNA is present in the RNA preparation. Proteins are unlikely to interfere with these dyes, but some detergents as well as phenol result in loss of fluorescence. Nucleic acid extraction reagents should thus be checked for effect on subsequent fluorescent assays.

Bisbenzimide fluorochrome dyes such as (2′-[4-hydroxyphenyl]-5′-[4-methyl-1-piperazinyl]-2,5′-bi-1H-benzimidazole) represent another option for measuring DNA. Researchers have studied the binding of these dyes to the minor groove of DNA and have found that sequences of adenine or thymine in the DNA sequence provide a minor groove dimension that binds the dyes best. Thus the fluorescent signal can show DNA sequence dependence, and the calibrant DNA should have a nucleotide composition that is similar to that of the DNA to be measured. These dyes are not as sensitive as cyanine dyes but are more sensitive than absorbance measurements. Low dye concentrations and high ionic strength are required in order for analysts to distinguish double-stranded DNA from RNA. Low ionic strength conditions are required in order to differentiate double-stranded DNA from single-stranded DNA.

**Detection by Size**

**AGAROSE GEL ELECTROPHORESIS**

Agarose gel electrophoresis provides a simple and accurate procedure for separating nucleic acids by fragment size. The technique can be adapted to separate fragments over a large range of sizes and can be used in a preparative or analytical fashion. For example, gel electrophoresis can be used to verify that a product of a PCR reaction is of the correct size. DNA fragments can be retrieved from a gel slice and provide a sufficiently pure PCR product for cloning or sequencing. The general integrity of an RNA preparation can be determined by gel electrophoresis as well. The stoichiometry of the nucleic acid fragment (tris-borate-EDTA) or TAE (tris-acetate-EDTA). TBE has a higher buffering capacity than TAE, but TAE should be used if the DNA is going to be retrieved from the gel. Denaturing RNA gels use MOPS Buffer (40 mM MOPS, 10 mM sodium acetate, 1 mM}

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EDTA, pH 7.0). Melting the agarose is conveniently achieved with the assistance of a microwave oven. The agarose will easily come to a boil, but this may not result in complete melting of the agarose, which may require bringing the solution to a boil several times, with intermittent mixing and holding periods, until the agarose is completely melted. Agarose particles transform from white to transparent before melting. Any partially melted agarose can be detected by swirling the flask while holding it up to the light. If the solution does not appear uniform, then it requires additional heating. The agarose is poured into the gel box after partial cooling but before setting up. Commercially available ready-to-use gels suitable for a particular application can also be used. For RNA-denaturing gels, formaldehyde is added under a fume hood to the melted agarose to a final concentration of 2.2 M or 6.7%. Before the agarose has hardened, the analyst places a comb in the gel to provide wells for the samples and size standards. Once solidified, the gel is placed in the electrophoresis box, and buffer is added until both sides are filled and there is a layer of buffer across the surface of the gel. Then 10X tracking buffer (40% sucrose with 0.25% bromophenol blue or 0.25% xylene cyanol or both) is added to each DNA sample to increase the sample density and to provide a tracking dye that is used to assess when the electrophoresis is finished. The increased density allows the sample to be transferred into the well and to remain there until it migrates into the gel during electrophoresis.

One or more lanes should be used for a DNA size standard containing fragments in the range that is relevant to the samples and agarose concentration. Size standards in various ranges are readily available. Bracketing the wells in size intervals is useful to determine the electrophoresis gradient has been uniform over the width of the gel. However, in the case of eukaryotic RNA preparations, the 18S and 28S ribosomal RNAs that are co-extracted from prominent bands (corresponding to 1900 and 4700 nucleotides) can also be used as size standards. In addition, the rRNA provides information on the RNA integrity because missing or fuzzy rRNA bands indicate problems with the quality of the RNA preparation. Once the wells are filled, the cover is placed over the gel box, and the box is connected to the power supply. The indicator dye in the tracking buffer added to the samples and size-standard allows the easy determination of how far the electrophoresis has proceeded. Bromophenol blue will migrate with DNA fragments of <500 base pairs, and xylene cyanol will migrate with fragments of 5000 base pairs.

The power supply is frequently run under conditions of constant voltage (1 to 10 V per cm) of gel length. Elevated voltage can cause high current, resulting in the generation of damaging heat and exhaustion of the buffer.

PULSED-FIELD ELECTROPHORESIS

This variation on agarose gel electrophoresis is used to separate a range of large DNA fragments and is most useful when resolution of 50,000 to 200,000 base-pair fragments is needed. The main difference is the addition of an alternating-field device that controls the power supply operating under constant voltage. Large fragments of DNA change conformation in order to move through the agarose pores, and the larger pieces take longer to readjust when the field is reversed and thus move more slowly than do smaller fragments. This allows resolution of fragments over the period of hours that the pulsed-field procedure operates. A commonly used ratio of forward to reverse is 3:1, and, in addition, the procedure typically calls for a stepwise increase in the unit time between reverses of the field. Electrophoresis may continue for 10 to 16 hours to avoid fluctuation in gel temperature, viscosity, and other properties that may cause artifacts.

POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

The format for performing PAGE is quite different from that for agarose gel electrophoresis. The general procedure for PAGE is described in Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis (1056). For resolution of small fragments of DNA in the 10 to 500 base-pair range, nondenaturing polyacrylamide gel electrophoresis is more suitable than agarose gel electrophoresis because separation of fragments of this size requires much smaller pore size than is achievable in agarose gels. The gel is prepared by polymerization of acrylamide monomers. The percentage of acrylamide dictates the range of fragment sizes that can be best resolved. For example, 20% acrylamide is suitable for the 10–100 base-pair range, and 5% acrylamide is useful in the 100–500 base-pair range. Commercially available ready-to-use polyacrylamide gels suitable for the particular size discrimination can also be used. The separated nucleic acids are visualized by staining with, for example, silver nitrate solution rather than with ethidium bromide or cyanine dye. However, staining with silver nitrate solution is laborious and time-consuming and not suitable for preparations that contain a large amount of protein, because proteins will also stain with silver nitrate.

CAPILLARY ELECTROPHORESIS AND LASER-INDUCED FLUORESCENCE (CE–LIF)

CEF has been used for many years to separate DNA fragments (for the general principles of CE, see Capillary Electrophoresis (1053)). The procedure relies on a principle similar to that underlying agarose gel electrophoresis. CE can utilize the cross-linked buffer systems applied in gel electrophoresis, but the technique can also use polymer-containing solutions (e.g., polymerization gels) that are designed to create pores that entangle proteins. These polymer solutions may be added to the capillary between injections, allowing a “fresh” gel prior to each run. In addition, capillaries can be used for more injections than are possible for polymerized gel-filled capillaries. The resolving power of the separation depends on the size of the pores, which is based on the composition of the gel. Kits are available to separate fragments into the desired size ranges. Fragment sizes outside the resolution window can possibly be separated, but the separation may not be reliable or reproducible when the gel capability is exceeded.

Fragments can be detected by a variety of mechanisms. Detection utilizing UV absorbance is possible, but the preferred and most common detection procedure is laser-induced fluorescence (LIF). Fluorescence offers improvements over UV detection in terms of selectivity and sensitivity. In addition, the detection limits for fluorescence are two to three orders of magnitude better than those for UV. Although DNA is intrinsically fluorescent, the background fluorescence and complex laser spectroscopy required preclude routine use. The most common way to label DNA is described in the section above on fluorescent protocols for RNA and DNA quantitation. This system is widely employed because of its simplicity (the dyes are added to the sample or into the reaction buffer) and effectiveness. The advantages of CE include speed of analysis, sensitivity using minimum sample volumes, and the potential for automation. These are achieved mainly by the inherent miniaturization of the gel. Automated
systems allow robust analysis of the quality, quantity, and fragment size of both RNA and DNA. CE applications have been especially important for evaluating the integrity of RNA because of the instability and progressive degradation of RNA caused by ubiquitous RNases, and new technologies that compare the ratios of 28S and 18S are improving the capabilities of these procedures.

**FILTER HYBRIDIZATION AND IN VITRO LABELING OF PROBES**

**Introduction**

Hybridization techniques were used early in molecular biology to identify individual nucleic acids and to estimate the degree of similarity between species. Hybridization is widely used in the procedures described in this and other chapters to visualize and identify nucleic acid sequences (see Nucleic Acid-Based Techniques—Amplification (1127), Nucleic Acid-Based Techniques—Genotyping (1129), and Nucleic Acid-Based Techniques—Approaches for Detecting Trace Nucleic Acids (Residual DNA Testing) (1130)). With the advent of restriction endonuclease digestion of DNA and electrophoretic separation by molecular mass, hybridization using labeled probes provided a way to visualize the organization of genes within a specific genome.

The hybridization techniques described are dot and slot blotting, Northern blotting, Southern blotting, in situ hybridization, and fluorescent in situ hybridization (FISH). All these techniques rely on the use of nucleic acid probes. Probes are oligonucleotides with specific DNA or RNA sequences that have been labeled with radioactive, fluorescent, chemiluminescent, chemical tags or enzymes (reporter molecules). Hybridized probes bind to complementary sequences on the target nucleic acids and are used to visualize and characterize targets, as described below.

**Dot and Slot Blotting**

Dot blotting is the simplest and quickest of the hybridization techniques. The nucleic acids are directly applied to a support membrane, which may be a nitrocellulose or nylon membrane, without prior separation of the nucleic acid species by agarose gel electrophoresis. The nucleic acids are spotted onto the filter using a micropipettor or an apparatus such as a dot blot or slot blot apparatus. This consists of a membrane frame with a membrane sandwiched in between the two pieces of the frame. The bottom frame plate is connected to a vacuum manifold, and the top piece of the frame has slots through which the nucleic acids are loaded. The samples are loaded under vacuum and pulled through the membrane by vacuum, with the nucleic acid binding to the membrane, and then the filter is air-dried. The nucleic acids are fixed to the filter either by heating to 80° for nitrocellulose membranes or by exposure to UV light for a predetermined time for nylon filters. Hybridization with a labeled probe provides confirmation of the identity of the nucleic acid but does not provide any information about the number or sizes of the species. The nucleic acid species of interest can be quantitated by spotting known concentrations of the purified nucleic acid on the filter and comparing the signal generated by the unknown samples with those of the standard preparations.

**Southern Blotting**

Southern blotting refers to the transfer of DNA from an agarose or polyacrylamide gel to a nitrocellulose or nylon membrane. Small, single-stranded DNA probes can then be used to visualize and identify the DNA species of interest. Southern blot analysis is based on a transfer and immobilization methodology developed in 1975, coupled with the electrophoretic separation of fragmented DNA. More specifically, the procedure typically is used to identify specific nucleic acid sequences in the context of a defined genetic topography, such as a restriction endonuclease map. The position of genes within the viral genome can be accurately mapped using a variety of restriction endonucleases in combination with Southern blot analysis. The procedure requires that DNA be obtained in sufficient quantity for analysis. Fragmented DNA is separated according to size using agarose gel electrophoresis. Double-stranded DNA fragments must be denatured before they are transferred and immobilized on a membrane by capillary action. The immobilized DNA is then cross-linked to the filter, which may be composed of nitrocellulose or nylon, as described above. However, the use of positively charged nylon membranes eliminates the need to fix the DNA to the nylon membrane. Nitrocellulose membranes are more fragile and may be probed up to 3 times with separate probes. Nylon membranes are more robust and may be probed 10 to 12 times, but they may present more background noise, particularly when they are used with chemogenic probes.

**Northern Blotting**

Northern blot analysis comprises a series of steps for the separation, transfer, and immobilization of RNA in a manner similar to the treatment of DNA using Southern blot analysis. Denaturation of the RNA is required to reduce secondary structure to ensure that the RNA separates in the agarose uniformly according to length. Denaturation of RNA is accomplished either prior to electrophoresis using glyoxal or dimethyl sulfoxide (DMSO) or during electrophoresis by means of gels that contain formaldehyde. Transfer is achieved in a manner identical to that used for Southern blotting. However, in the case of Northern blotting, it is unnecessary to denature the RNA prior to transfer because denaturation is accomplished before electrophoretic separation of the RNA species. The immobilized RNA is cross-linked to the membrane in a manner similar to the cross-linking of DNA.

**In Situ Hybridization and Fluorescent In Situ Hybridization (FISH)**

Hybridization of a nucleic acid in situ classically refers to determining the location of that nucleic acid sequence in its natural state—in tissue, in individual cells, or on a chromosome. In situ hybridization probes are designed to bind to complementary nucleic acid sequences, whether they be DNA or RNA. The purpose of these hybridization procedures is to discover where in a
obtaining the final sequence information includes all processes associated with sample preparation, sequencing, data assembly, and their purification has become routine, and high-quality synthesis and purification are commonly achieved. Moreover, larger segments can be synthesized, and even larger segments are required, the subsections can be designed for concatenation and ligation.

Custom synthesis of DNA oligonucleotides is readily achievable in the laboratory using commercially available reagents and equipment. Alternatively, probes can be custom ordered from numerous commercial providers. Size-exclusion procedures for purification generally are used to eliminate incomplete oligonucleotides. RNA oligonucleotides also may be chemically synthesized or generated in vitro using complementary cloned DNA fragments under the control of various prokaryotic RNA polymerase promoter sequences. The use of DNA probes is much more common, but there may be some applications in which the increased association of RNA–RNA or RNA–DNA hybrids is advantageous.

The principal procedures of labeling DNA are direct labeling using a kinase reaction to attach a labeled nucleotide to the end of each DNA strand, by incorporating labeled nucleotides into a nicked DNA by utilizing the DNA repair function of the Klenow fragment of Escherichia coli DNA polymerase I enzyme (nick translation), and by PCR. This last procedure generates a relatively higher yield of internally labeled probe because each round of thermal cycling doubles the amount of labeled probe, whereas the former procedures result in a ratio of less than one probe molecule per template molecule. The PCR procedure also is used to generate unique probes with a variety of moieties located at the termini.

NUCLEIC ACID SEQUENCING

Introduction

The first DNA sequencing procedure, described in 1977, utilized chemical cleavage to specifically introduce chain breaks in a DNA sequence (Maxam and Gilbert sequencing). The procedure proved to be of significant utility in the early years of molecular biology, but it has not been used to perform high-volume sequencing and therefore is not discussed in detail here. The majority of sequencing performed today is based on the dideoxysequencing procedure, also described in 1977 (Sanger sequencing). This procedure fundamentally changed sequencing by exploiting the enzymatic specificity of polymerases that introduce strand interruptions at specific bases. This is the most widely recognized sequencing procedure and is considered a routine assay in molecular biology laboratories. Innovations in instrumentation, sample preparation and collection, data management, data analysis, and sequence assembly have relied on this sequencing procedure as their fundamental sequence generator.

High-throughput sequencing takes all the elements of the sequencing procedures and applies them to a mass collection of sequence data, typically for larger genomes, but high-throughput sequencing certainly may be used for smaller projects as well. Obtaining the final sequence information includes all processes associated with sample preparation, sequencing, data assembly, and data finishing. The technology to achieve these individual objectives includes the instrumentation, disposables, protocols, and procedures.

Sequencing Reaction

The dideoxysequencing procedure takes advantage of specificity of the Klenow enzyme to introduce chain-terminating nucleosides, called dideoxynucleotides, intermittently during the polymerase extension process. The sequencing of each sample requires four separate reactions (one for each base). The resulting mixture of various nucleotide chain lengths is then separated.
on the basis of individual molecular masses. The incorporation of radioactively labeled nucleotides during the sequencing reaction permits the detection of the nucleotide chains.

Improvements in biotechnology have led to the discovery of more robust enzymes with high fidelity, improved stability, and other attributes that have led to longer reads and improved sequence fidelity. These improvements have made possible the introduction of cycle sequencing, which is now commonly used. The principle of the cycle sequencing procedure is a combination of Sanger sequencing and aspects of PCR amplification, whereby dideoxynucleotides are incorporated into the amplified DNA. Cycle sequencing leads to a higher concentration of labeled fragments covering a wider range of sizes than does Sanger sequencing, leading in turn to a higher read length.

Separation Procedures for DNA Sequencing Fragments

The previous sections of this chapter deal with the treatment of intact DNA and RNA molecules; the following sections address the challenges of separating the fragments that result from the sequencing reactions, notably slab gel sequencing and capillary electrophoresis. Subsequent sections address detection technologies and sequence integrity.

Slab Gel Sequencing

Polyacrylamide gel electrophoresis, frequently referred to as slab gel electrophoresis, was the first separation mechanism employed for the separation of DNA sequencing fragments. As described above, the electrophoretic separation of DNA fragments is driven by the size of the fragments in the reaction mixture. However, for slab gel sequencing the pore sizes are chosen so that single-base resolution for many hundreds of bases is possible. In addition to the polyacrylamide in the gel, a denaturant such as urea is frequently included to ensure denaturation of the fragments. Until the implementation of multicapillary sequencing systems, the separation power and throughput of slab gel separation mechanisms were often considered state of the art.

Capillary Electrophoresis Sequencing

As noted above, capillary electrophoresis offers significant advantages over gel-based separations. However, as with slab gel sequencing, the pore sizes are chosen so that single-base resolution for many hundreds of bases is possible. Multicapillary systems that utilize 8 to 384 capillaries are commercially available. These systems are the primary systems used for large-scale DNA sequencing, and, theoretically, they yield more than 1.1 billion base pairs of DNA sequences per year.

Detection

RADIOACTIVITY

The first detection strategies for DNA sequencing reactions utilized radioactive isotopes such as $^{32}$P or $^{35}$S, primarily because these were practical for gel separations. The advantages are that detection is universal, low limits of detection are possible, mobility shifts are eliminated, and fidelity differences for the DNA polymerases do not occur. Disadvantages include the high disposal and safety costs, the inability to multiplex (ultimately limiting throughput), and the need for 24 to 36 hours of exposure time (i.e., no real-time detection).

FLUORESCENCE

Fluorescence dyes have largely replaced radioactive isotopes as detection tools during DNA sequencing, mainly because they do not have the disadvantages of radioactive probes. Because the dyes can be discriminated by means of their emission maxima, multiplexing is possible, so four sequencing reactions per sample can be replaced by a single reaction using four different labels. Thus a single lane can be used rather than the four separate lanes that were necessary with radioactive probes. Additional advantages are higher throughput and automated data collection in real time.

MASS SPECTROMETRY

Mass spectrometry (MS) has revolutionized the field of biochemistry and has significant potential in the area of nucleic acid sequencing. Soft-ionization techniques such as electrospray ionization and matrix-assisted laser desorption–ionization have expanded the potential application of MS to DNA sequencing. MS offers some advantages over other detection methodologies, including speed of fragment detection (signal acquisition is in the range of microseconds versus hours for conventional approaches) and accuracy (e.g., the molecular mass of each fragment can be determined with a high degree of accuracy). The Sanger procedure makes use of mass differences of the fragments generated as part of the polymerization reaction. MS is sufficiently precise to resolve fragment sizes that differ by only one base pair. Unfortunately, the sensitivity of MS detection suffers as fragment length increases, and the 100-base-pair barrier has yet to be crossed.

More recently, other sequencing technologies have emerged that are based on massively parallel sequencing techniques that attempt to achieve low-cost sequencing. These techniques are based, for example, on solid-phase sequencing or they make use of highly parallel and miniaturized pyrosequencing, which is described in Nucleic Acid-Based Techniques—Genotyping (1129).
Sequence Integrity

A prerequisite for automated data collection and interpretation is that the data must be of good quality, which means minimizing human intervention and allowing the system to make base identifications following detection steps. It is a critical step to ensure accurate base identification by minimally sequencing both strands of the DNA several times. In addition, other tactics may be employed, such as using primers at different sequence positions, which can improve the accuracy of the developed consensus sequence. This task can be facilitated by the use of specialized software packages that are commercially available. More recent technology developments have produced alternative sequencing platforms that are more amenable to large-scale sequencing projects. These technologies include array-based platforms on which short stretches of target are sequenced on a chip that supplies raw data to sophisticated computational programs that reconstruct the sequence. Other sequencing approaches have been developed for the rapid sequencing of short nucleic acid sequences such as oligonucleotides of short PCR products. These technologies include MS-based and pyrosequencing platforms, the latter of which is described in Nucleic Acid-Based Techniques—Genotyping (1129).

(1127) NUCLEIC ACID-BASED TECHNIQUES—AMPLIFICATION

INTRODUCTION

The basic principles of nucleic acid amplification technologies (NAT) and definitions of the various techniques are covered in Nucleic Acid–Based Techniques—General (1125). The current chapter covers major techniques that result in amplification of targeted nucleic acid sequences. The most common NAT assay is the polymerase chain reaction (PCR), which was first described by Kary Mullis. This procedure has been further refined to amplify a DNA fragment starting from RNA (reverse transcription-PCR, or RT-PCR). Initially, PCR was used in a qualitative manner to amplify and detect DNA molecules because its exquisite sensitivity paired with its high specificity made it a useful tool for the detection of nucleic acid targets. Since its inception, the number of PCR applications has expanded rapidly, and the technique, which now includes quantitative and multiplex assays, is currently used in almost every field of research and development in biology and medicine. In addition to the changes and improvements to the original design of the PCR procedure, alternatives to PCR are techniques used to amplify target nucleic acids to generate RNA instead of DNA amplics. The most commonly used technique is nucleic acid sequence–based amplification (NASBA) and the transcription-mediated amplification (TMA) which are described here in detail. In contrast to PCR, which relies on incubating the sample at three different temperatures, NASBA and TMA are based on isothermal conditions.

In addition to amplification of the target nucleic acid, the amplification step also can be directed at the signal used for detection (signal amplification). The most commonly employed technique is the branched DNA (bDNA) assay, in which the signal, typically a fluorescent probe that binds to the target sequence, is amplified. The bDNA assay is used predominantly for viral nucleic acid detection and quantitation.

This chapter describes the main assay components necessary for a PCR procedure and includes a discussion of the general optimization of PCR assays. The various PCR assay formats, including PCR, nested PCR, and RT-PCR are covered, and a discussion of the detection of the resulting amplicons follows. Although all these assays are essentially qualitative procedures, they can be modified for semiquantitation, and the various modifications are described. For accurate and reliable quantitation, real-time PCR has now replaced the methods listed above; real-time PCR and real-time RT-PCR are described in the NAT Assays section. The same section includes a discussion about probes and dyes that are an essential component of real-time PCR and the methods of quantitation using the generation of standard curves. The next PCR technique discussed is multiplex PCR, which is used for simultaneous detection of multiple targets or for normalization of assay results. Apart from PCR, the major alternative NAT tests that are used routinely, primarily in blood screening and clinical diagnostic screening are NASBA and TMA. The final technique described is whole-genome amplification, wherein the complexities of amplification require modifications to the PCR procedures. The chapter concludes with a discussion about the evolution of instrumentation used in NAT assays and the quality assurance and quality control issues associated with NAT because this is probably one of the most highly regulated biological techniques, especially when applied to blood screening.

ASSAY COMPONENTS

Enzymes

The essential components for NAT assays—polymerases, reaction buffers which include deoxynucleotides, ions, primers, probes, and fluorescent dyes—can be chosen from a broad selection of commercially available NAT reagent kits and vendors. Polymerases suitable for NAT applications can, in principle, be grouped into Taq DNA polymerases or DNA I polymerases from other Thermus species that are polymerases with features that are similar to those of Taq DNA polymerase. In addition, so-called proofreading polymerases are available (e.g., from Pyrococcus species) that display a 3′→5′ exonuclease activity capable of removing wrongly incorporated DNA bases from the growing DNA strand under amplification conditions. Taq DNA polymerase is the standard NAT enzyme and is the one most often used in NAT assays. Modifications of Taq DNA polymerase, such as deletions of the 5′→3′ exonuclease domain (Klenow fragment, Stoffel fragment) or point mutations for improved incorporation of deoxynucleotides are also employed (e.g., for PCR-based sequencing reactions). Proofreading DNA polymerases or mixtures of Taq DNA polymerase with a proofreading polymerase are used if either fidelity of the NAT product is critical (e.g., for DNA cloning experiments) or longer NAT products are to be amplified. For RT–PCR, a reverse transcriptase is necessary to first convert the RNA target to copy DNA (cDNA) that can subsequently be amplified. For TMA reverse transcriptase with an RNase H activity is needed to convert the RNA target to double-stranded template DNA, while for NASBA exogenous RNase H
is added to the reaction mixture. Depending on the reaction environment, two types of enzymes can be used to generate cDNA: a reverse transcriptase isolated from retroviral sources or a DNA polymerase that can function both as reverse transcriptase and DNA polymerase. Finally, chemical modification of the polymerase, resulting in an inactive enzyme at temperatures below 90°, is now typically used to prevent mispriming of templates at sub-optimal temperatures (see section on Assay Optimization).

**Reaction Buffers**

Reaction buffers vary with respect to ion composition, pH, and additives and are sometimes specifically adopted for particular applications such as multiplex PCR, real-time PCR, RT–PCR, TMA and NASBA. An important component of the reaction mixture is Mg²⁺ ions, or, in the case of polymerases with both reverse transcriptase and DNA polymerase functions, such as *Thermus thermophilus* (*Tth*), Mn²⁺ ions. Other additives that enhance the sensitivity and specificity of the assay may be present. The concentration of the four deoxynucleotide triphosphates (dNTPs) must be optimized.

**Primers**

Primer sets are oligonucleotides with sequences that are designed specifically to prime the amplification of a portion of a target nucleic acid of interest. Synthetic oligonucleotide primers for both standard PCR and for real-time or quantitative PCR are designed for the specific recognition of and binding to a single DNA or RNA sequence. Such specificity is achieved through design that involves both the length and the sequence of the primers. Length and sequence specifications have separate criteria that must be simultaneously met in order for the primers to perform properly. The length of a primer is a statistical issue that relates to the issue of the minimum length of a specific sequence necessary to guarantee that the desired target sequence is unique, regardless of the size or complexity of the genome. As an example, in the case of the human genome, with its 3.2 billion DNA bases, that length is 17 bases. For this reason the vast majority of PCR primers are between 20 and 25 bases long. The specificity of a primer should be determined by comparison with sequences in all known databases. Tools available on the Web facilitate such comparisons.

In terms of primer sequence, the issues to consider are $T_m$ (the temperature at which 50% of the double-stranded nucleic acid molecule becomes single-stranded) and secondary structure. Every DNA has its own characteristic $T_m$, determined by length, sequence composition, and reaction environment. PCR primers are designed to bind to a perfectly complementary DNA sequence via guanine-cytosine (G-C) and adenine-thymine (A-T) base pairing. The $T_m$ of the two PCR primers used in a reaction should be as close as possible. In terms of secondary structure, the formation of secondary structures by intra- or intercomplementarity should be minimized. Interaction between different primers can result in primer–dimers that will compromise assay sensitivity and specificity. All of the design issues presented are accounted for in any one of the dozens of primer design software packages that are available and can be found on the Internet.

**Assay Optimization**

NAT assay optimization is necessary for successful amplification that is sensitive and specific. Parameters that should be optimized include the thermocycling conditions, both temperatures and cycling times (that depend to a large extent on the target, primer, and probe sequences), concentrations of template, concentrations of NAT reagents, sample matrix and the number of amplification cycles. In the case of multiplex PCR, a compromise among elements of the reaction conditions is usually necessary because of the difficulties in optimizing the conditions for all the primer and probe sets. Recent changes have been made to improve sensitivity and specificity of NAT assays. One change is hot-start PCR, in which the addition of one of the essential components of the NAT assay, typically the DNA polymerase, is temporarily withheld. When this occurs during reaction setup, the initial nucleic acid denaturation step prevents nonspecific amplification due to mispriming at suboptimal temperatures. Early hot-start procedures made use of wax barriers that effectively separated essential components into two liquid phases that were mixed only when the wax melted. However, this procedure has been replaced by two important hot-start technologies that do not require physical separation of the components by inconvenient additional handling steps. In the first procedure, antibodies directed against the DNA polymerase are complexed with the enzyme and lose their binding avidity at elevated temperature at the start of the reaction. The second procedure uses chemical modification of the polymerase, resulting in an inactive enzyme. At temperatures above 90°, typically in the first denaturation step, the modifier dissociates from the enzyme, and the enzymatic activity is restored. The advantage of an antibody-mediated hot start is the immediate release of enzyme activity at the start of the reaction by a very short heat incubation step. However, antibody-mediated hot-start chemistries tend to be less stringent when compared with chemically activated enzymes if there is a large excess of active polymerase molecules.

**NAT ASSAYS**

This section describes the basic techniques of PCR, nested PCR, and RT-PCR and procedural modifications that allow semiquantitation.

**Polymerase Chain Reaction**

The PCR technique is based on a three-step process: denaturing double-stranded DNA (dsDNA) into single strands (ssDNA), annealing primers to the ssDNA, and enzymatic extension of primers that are complementary to the ssDNA templates. Each step is usually carried out at a different temperature. By cycling the temperature steps many times (usually 30 to 45 times), a billion-fold amplification of the target nucleic acid can be achieved, but the optimal number of cycles should be determined empirically. In some cases, especially where sensitivity is more important than false positive results due to excessive cycling,
such as in blood screening, extra sensitivity can be gained by increasing the number of cycles to 60 to ensure that extremely low levels of target are detected. In a typical reaction, PCR product (amplicon) doubles at each cycle of amplification (exponential amplification). The increase in amplification in the early cycles follows a sigmoidal curve. In later cycles, the concentrations of the template strands and amplicons favor template strands re-annealing instead of PCR primer annealing to the template. At this point the concentration of the PCR product no longer doubles after each cycle, and the curve begins to plateau. A thermostable enzyme such as Taq-polymerase is a prerequisite because temperature cycling at 95° (the typical temperature step used to denature double-stranded templates) would inactivate a thermolabile polymerase.

NESTED PCR

An early variation of the PCR assay was nested PCR, which was designed to increase the assay’s sensitivity and specificity. In this procedure amplicons from the initial PCR reaction are subjected to a second round of amplification using a different set of primers. This set of primers is specific to the amplicon sequence but is within the first set of primers (nested primers). The advantage of amplification with two sets of target-specific primers is increased specificity (any nonspecific amplification during the first amplification round would be reduced) and increased sensitivity (due to initial amplification of the target in the first amplification round). In addition, amplification of a product of the expected size is taken as confirmation of the presence of the target. However, a major drawback of this procedure is the high likelihood of cross-contamination due to the increased manipulation of amplicons generated in the first round of amplification. The use of highly specific primers and probes and the optimization of reaction conditions have resulted in the diminished applications of this procedure for routine testing, but the procedure is sometimes used for samples that are difficult to amplify by conventional PCR.

RT-PCR

In amplifying RNA targets, analysts prepare cDNA before the amplification step (RT-PCR). One-step and two-step RT-PCR procedures are available. In one-step RT-PCR the reverse transcription of RNA into cDNA and the subsequent amplification step are carried out in a single reaction without intermediate procedures. Therefore the reaction mixture for one-step RT-PCR includes the gene-specific amplification primers that are used for both reverse transcription and amplification. The advantage of this procedure is the overall reduction in handling time, increased throughput, and reduced contamination risk because reopening the reaction vessel is not necessary. In contrast, in two-step RT-PCR the reverse transcription and amplification are performed as two separate steps. In general, random primers or oligo-d(T) primer rather than gene-specific primers are used for the reverse transcription step. An aliquot of the cDNA synthesis reaction is then transferred into the NAT reaction for subsequent amplification. The advantage of this procedure is the standardization of the reverse transcription reaction, which can be used as a single source for the analysis of multiple transcripts in gene expression analysis.

DETECTION OF AMPLICONS

Following amplification, analysts can employ a variety of procedures for detection of the amplicon as described in detail in the general information chapter, Nucleic Acid–Based Techniques—Extraction, Detection, and Sequencing (1126). These include agarose gel electrophoresis with ethidium bromide or other dyes, capillary electrophoresis, and laser-induced fluorescence and hybridization followed by chromogenic detection such as streptavidin horseradish peroxidase detection, chemiluminescence, or fluorescent detection using labeled probes.

Quantitation—The original PCR and RT-PCR assays were qualitative and detected amplicons at the end of the reaction. Such detection is not easy to quantitate because at this stage the amplification is in a plateau phase at the end of the assay, and the amount of amplicon is not necessarily directly related to the quantity of the starting template. Several approaches have been deployed to attempt to overcome the shortcoming of PCR to produce reliable, quantitative results. Initial attempts at quantitation relied on assessing the amount of amplified DNA during the early or exponential part of the assay, but this procedure was fraught with problems because the aliquots had to be taken from the reaction mixtures at regular intervals, thus greatly increasing the risk of cross-contamination. One of the earliest and most straightforward approaches to quantifying PCR products was to measure the amount of amplicons that were generated during the exponential phase of the reaction by comparing this to a serially diluted external control. Several aspects, including variability in sample preparation and variations in reaction conditions, however, hampered this approach. Because of the exponential amplification of NAT procedures, even small errors or variances can lead to distinct differences.

Compared with dilution procedures, competitive PCR proved to be a much more precise approach to achieving reliable estimates of the originally present target molecules. This procedure relies on the simultaneous co-amplification of a specific target sequence in the presence of increasing concentrations of an exogenous target molecule (control) which shares the primer binding sites with the target sequence but whose sequence is slightly modified or shortened in order to facilitate discrimination from wild-type amplicons. In addition, the concentration of the control is known. The close sequence homology and similar size of the control and target amplicons are designed to ensure that the template and internal control are amplified with comparable efficiency. The relative strength of the amplicon bands of template and control can be assayed, for example, on ethidium bromide–stained agarose gels, giving a relatively precise quantitation of the wild-type target. A drawback of this approach is that the control and template should be present in the reaction in approximately the same quantity in order to yield correct results. The development of real-time, quantitative PCR has eliminated the variability associated with quantitative PCR, thus allowing the routine and reliable quantification of PCR products.

REAL-TIME PCR AND REAL-TIME RT-PCR

Although gene quantitation by quantitative PCR was a widely used procedure, its applications were expanded by the advent of real-time PCR and real-time RT-PCR. Real-time PCR displays the same advantages as standard quantitative PCR—sensitivity, specificity, and a wide dynamic range—but the real-time procedure offers the additional advantage of requiring no post amplification processing because it combines amplification and detection in a single step. Real-time PCR collects data
throughout the amplification process by measuring a fluorescence signal created as amplification progresses. A multitude of fluorescence chemistries allows the correlation of generated PCR product to fluorescence intensity. In principle, fluorescence intensity will increase with every cycle performed. Once the intensity is greater than background fluorescence, the so-called cycle threshold (Ct) value is achieved. This value, which represents the first cycle in which there is a detectable increase in fluorescence above the background level, is used to measure relative or absolute target quantities. The Ct value is inversely proportional to the number of target molecules in the sample and thus provides a means to quantitate the amount of target in the starting material (i.e., the greater the number of target molecules present, the lower the Ct value).

The reaction conditions for real-time PCR applications have to take into account the presence of the probe(s) and will require optimization. The most commonly used probes currently are hydrolysis probes, although hybridization probes are an alternative. In most cases, the amplification and detection steps can be combined into a two-step cycling reaction, but these conditions have to be optimized. In contrast, DNA-binding dyes which may also be used for amplicon detection require separation of the annealing and extension steps since the dye binding occurs during the extension step which is usually done at 72°C.

A fluorescent DNA intercalating dye is used for detection of the PCR product in real-time mode. This dye emits light when bound to double-stranded DNA and the subsequent increase in fluorescence can be detected by real-time PCR instruments. Dyes that bind to dsDNA bind not only to the specific PCR product but also to artifacts such as nonspecific PCR products and primer–dimers. Analysts have observed substantial differences in the specificity of dsDNA-binding dyes in use with real-time PCR kits. Therefore, some analysts recommend verifying the presence of a single PCR product by gel electrophoresis to determine the correct size of the PCR product. Also, a melting curve analysis is advisable to ensure the absence of artifacts that could contribute to the fluorescent signal and thereby lead to misinterpretation of quantitative data. Alternatively, sequence-specific labeled probes can be employed. A wide variety of fluorescence-labeled probes and primers exist for use in real-time PCR and are described in the next section.

Real-Time PCR Probes—The difference between conventional PCR and real-time PCR is the presence of a third chemically synthesized oligonucleotide, the probe, which, for the most basic hybridization probes, contains some type of reporter molecule, usually a fluorescent molecule or fluorophore. Non-nucleic acid materials can be added to chemically synthesized DNAs that are then incorporated into oligonucleotide probes for real-time PCR. Other applications include hybridization probes such as those used for fluorescence in situ hybridization (FISH) and microarrays and probes designed to capture other nucleic acids. A challenge arises in using fluorescent probes for real-time PCR because the unbound or free probe is not removed before detection, thus requiring a means to distinguish between signal obtained from bound and free probe. In contrast, FISH assays involve washing away free probe following hybridization.

All of the issues associated with primer design for conventional PCR apply to real-time PCR primers as well as to the probe sequence. As a general rule only two additional considerations apply to the probe sequence. One of these is thermodynamic, and the other specifically concerns the reporter moiety itself. Thermodynamically, a good probe molecule that is designed to bind to the probe sequence such that the two PCR primers will have a Tm that is about 5° higher than that of the two primers. In the large majority of cases the amplicon will be between 100 and 500 DNA bases in length, although for real-time PCR a smaller amplicon between 100 and 150 bases long results in a more efficient reaction. Thus it is rarely a problem to find a sequence inside a PCR amplicon that meets the necessary criteria.

Current probe designs overcome the problems of background from unbound probe using simple hybridization probes. In the original design, two probes that hybridize to adjacent sequences on the target nucleic acid are labeled. The reporter moiety is a fluorescent molecule attached to the 3' end of the upstream probe sequence, and a second fluorescent molecule is attached to the 5' end of the second probe. Excitation of the 5' fluorophore with light energy of the proper wavelength results in absorption of that energy, followed by emission of light energy of a slightly longer or less energetic wavelength (Stoke's Law). This emitted energy then excites the 3' fluorophore if it is close enough to the emitter and compatible with it in the sense that the emitted energy from the 5' fluorophore can excite the 3' fluorophore. When this occurs, the observed fluorescent light wavelength will be that of the acceptor molecule and not that of the donor. Fluorescence absorption and emission spectra are readily available for all of the commonly used fluorophores, and the only applicable rules are that the two fluorescent molecules must be fewer than 40 DNA bases apart and that the emission spectrum of the donor must overlap the absorption spectrum of the acceptor. Thus hybridization of the two probes, also known as hybridization probes or FRET probes (Fluorescence Resonance Energy Transfer), results in the emission of a fluorescent signal by the acceptor, and the latter signal can be detected. In the absence of hybridization, the probes are sufficiently separated in solution so that energy transfer cannot occur, and only background fluorescence is emitted by the donor.

Issues of fluorophore compatibility have been resolved by the increased use of a special class of molecule called a quencher. Quenchers are fluorescent molecules that absorb fluorescence energy over a wide range of wavelengths. Instead of re-emitting that energy as light they simply dissipate it as heat. Thus, if a quencher molecule is placed at the 3' end of a probe and a fluorophore at the 5' end, the probe will remain dark even when excitation energy is present so long as the molecule remains intact (hydrolysis probes). These probes utilize the 5' nuclease activity of the DNA polymerase to hydrolyze a probe bound to its target amplicon. Cleavage results in separation of the reporter and quencher and permits fluorescence of the reporter. This reduces much of the work of optimization of the assay conditions (since only a single probe is used) and background noise is a conformational change that forces the quencher and reporter apart, permitting fluorescence of the reporter. A variation on these kinds of probes is a combined primer and probe in which, again, the quencher and reporter are in close proximity in the native probe, but resulting in no signal. Priming and subsequent elongation of the primer–probe results in hybridization to the newly synthesized DNA strand, causing spatial separation of the quencher and reporter and resulting in the generation of a signal.

Probe Labeling—Modern synthetic oligonucleotide modification chemistries permit the manufacture of oligonucleotides with non-nucleic acid materials. Placement of modifications is carried out in one of two ways: during synthesis or after synthesis. For the former, modifications are constructed in such a way that they behave like the four DNA or RNA bases that are routinely...
placed in the sequence. The modification is then presented in the desired location during the synthesis as if it were just another base in the series. In the latter, usually employed when more than one modification occurs, the synthesis contains a linker, such as a linker group, to which the desired modification is then attached. This process is often called “hand-tagging.”

Perhaps the best-known example of hand-tagging is the conventional dual-labeled probe used in real-time PCR. The quencher is placed at the 3’ end of the sequence during synthesis, and the fluorescent reporter molecule is hand-tagged to an amino modification at the 5’ end of the sequence after the synthesis is finished and has undergone purification. Some modifications, such as biotins, are designed so that multiple modifications can be carried out in a single synthesis. Thus, it is possible to modify a synthetic DNA or RNA sequence to contain a number of different non-nucleic acid molecules. A cost is associated with such modifications insofar as alterations often are achieved with a loss of mass due either to an inherently lower efficiency of modifications to bind to the oligonucleotide as compared with standard DNA or RNA bases or to the requirement that the synthesis must be purified before modification, after modification, or both.

The benefits of modifying synthetic DNAs or RNAs usually outweigh the costs. The standard, quenched, dual-labeled, real-time PCR probe has permitted precise quantification of gene expression. Fluorescently labeled DNA oligonucleotides are also essential components of in situ hybridizations and microarrays. Some modifications confer increased thermal stability when synthetic DNAs or RNAs are hybridized to complementary DNAs or RNAs by comparison with unmodified DNA—DNA and DNA—RNA duplexes. These analogues include peptide nucleic acids, 2′-fluoro N3-P5′-phosphoramidates, and 1′, 5′-anhydrohexitol nucleic acids. Although such analogues succeed to varying degrees in achieving increased thermal stabilities, they fail to provide enhanced target recognition. Another approach is to use base analogues such as locked nucleic acid, which is an analogue that contains a 2′-O, 4′-C methylene bridge. This bridge restricts the flexibility of the ribofuranose ring and locks the structure into a rigid bicyclic formation, conferring enhanced hybridization performance and stability.

The modification of a probe typically is governed by its intended use. Generally, fluorescent reporters are used in real-time PCR and for in situ hybridization. The range of available fluorescent reporters covers the spectrum from 517 nm to 778 nm. For hybridization probes, base modifications are preferred because these primarily alter thermodynamic interactions between bases, leading to improved specificity. Amino attachment groups, both with and without C-spacers, are used to attach other modifications to DNA sequences and to attach DNA sequences to solid surfaces such as glass slides. An example is the attachment of biotin molecules to DNA sequences. Biotin forms a strong bond with streptavidin-coated materials such as magnetic beads, allowing capture of specific nucleic acids that may themselves be hybridized to other molecules.

**Quantitation**—PCR products may be quantified using a standard curve drawn from replicative serial dilutions of a reference reagent or standard for the nucleic acid sequence of interest. The concentration of the nucleic acid in the reference reagent is known. Real-time PCR quantitation based on a standard curve may utilize plasmid DNA or other forms of DNA. However, the efficiency of PCR must be the same for the standards and the target samples. Performing PCR from purified targets can in some cases be more efficient than performing PCR with complex nucleic acid mixtures. The cycle threshold (Ct) values and concentrations of the dilutions of the reference reagent can be used to construct a standard curve from which the concentration of the unknown sample can be estimated. When the assay run conditions have been well standardized and the standard curve for a particular target has been well calibrated, in subsequent assay runs it may be sufficient to co-amplify only two dilutions of a reference reagent (usually dilutions containing known amounts of nucleic acid at high and low concentrations). These dilutions, or calibrators, can then be used to quantify any unknown samples by comparison of the C, values.

**Multiplex PCR**—Multiplex PCR describes the simultaneous amplification of several nucleic acid targets in a single assay reaction. This is a particularly demanding variation of PCR because it requires the use of a single set of reaction conditions for the amplification of multiple targets with different sequence characteristics. Additional complications can arise due to the increased chance of nonspecific amplification products arising from multiple primer interactions. In addition, the differing individual target amplification efficiencies can result in weaker reactions being out-competed by stronger, more efficient reactions.

Both qualitative and quantitative applications of multiplex PCR have been described in the literature, as have multiplex RT-PCR assays. Quantitative multiplex PCR relies on either the generation of multiple standard curves to enable quantitation of each target in the assay, or the inclusion of internal competitor sequences that can be used as calibrators.

Hybridization kinetics of primers and probes may be significantly different, even when designed using the same algorithm. This leaves the analyst with very limited room to optimize reaction conditions. However, optimization may include adjustment of DNA primerase amount, Mg++ to increase hybridization efficiency, or primer concentration. Especially in real-time PCR, optimization of primer concentration is critical for quantitative co-amplification of target genes. These are contained in the sample at significantly different amounts. Increasing hybridization efficiency of the primer–probe system can be achieved by providing sufficient reagents, such as Mg++, as well as adding a “molecular crowding” reagent that increases the effective concentration of all reaction components in the mixture. Multiplex PCR is not only used for genotyping applications, but also for quantitative real-time PCR because it offers several advantages over standard single real-time PCR reactions. Some of these advantages are a minimized amount of sample used, increased precision through the use of an internal control (e.g., housekeeping gene) co-amplified with the target gene in the same reaction, no separate pipetting steps, and cost-effectiveness.

Most PCR assays, however, suffer from a common problem—that of minimizing differences in extractions or amplifications between different samples. Multiplex PCR is useful in cases where it is critical to ensure that variability in quantitation of different samples is not due to differences in nucleic extraction or amplification measurements (usually when one measures the production of an mRNA species). Certain precautions and techniques can be employed to minimize these challenges; they are discussed in the next section on normalization of assay results.

**Normalization of Assay Results**—To minimize the effects of assay variables, analysts sometimes use a relative quantitation procedure that normalizes the target transcript level to a control that can be employed and compared for all samples included in the gene expression study. Probably the most reliable and most frequently used relative quantitation procedure relies on the measurement of “housekeeping” or control genes to normalize the expression of the target gene in a multiplex PCR format. This procedure is preferred because the quantitation of both the housekeeping gene and the target gene are influenced by variations in cDNA synthesis efficiency, the presence of the inhibitors contained in the sample. However, it should be noted that the efficiency of conversion of target RNA to cDNA is not necessarily consistent even within a single-tube reaction but is a function of primer design, target sequence, etc. which may differ between target and housekeeping genes. The selection of appropriate control genes can cause problems because they may not necessarily be equally expressed across all unknown

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samples and may vary under experimental conditions. Normalizing measurements to a set of housekeeping genes in order to avoid the problem of variability may circumvent this concern. Alternatively, analysts can establish a thorough evaluation of housekeeping genes that do not alter gene expression levels under the experimental conditions.

All the NAT techniques described thus far are variations on the PCR assay, which is the most widely used of the NAT techniques. However, isothermal assays that are based primarily on the amplification of RNA are used for routine purposes. This is known as the transcription-mediated amplification (TMA) assay, which is closely related to the nucleic acid sequence–based amplification (NASBA) assay. Both assays are described in more detail in the following section.

NUCLEIC ACID SEQUENCE-BASED AMPLIFICATION AND TRANSCRIPTION-MEDIATED AMPLIFICATION

Both NASBA and TMA rely on in vitro isothermal amplification for detection and amplification of nucleic acids, also referred to as self-sustained sequence replication or 3SR. The major difference between the assays is that NASBA uses three enzymes—reverse transcriptase (RT), RNA polymerase, and RNase H—whereas TMA uses only two enzymes; RT and RNA polymerase. The complete procedure generally is performed at 41° to 42° using two primers. Both NASBA and TMA are especially suited to amplifying RNA analytes, including rRNA, mRNA, pathogens that have RNA as their genetic material, as well as DNA targets.

One of the primers that has a promoter sequence for the RNA polymerase at the 5′ end binds to the RNA target and is extended via the DNA polymerase activity of the RT. The product of this reaction is an RNA–DNA hybrid. RNase H activity then specifically digests the RNA strand of the hybrid, leaving only the cDNA to which the second primer can bind. A complementary strand of DNA is then synthesized by the RT, resulting in a dsDNA molecule with a T7 promoter at the 5′ end. The T7 RNA polymerase then transcribes multiple copies of the RNA amplicon. The RNA copies may undergo the same cycle to create new amplicons preferentially without any further shortening of the fragment length. PCR-based WGA amplification tag sequences are amplified preferentially without any further shortening of the fragment length. PCR-based WGA amplification (NASBA) assay. Both assays are described in more detail in the following section.

WHOLE GENOME AMPLIFICATION

Historically, whole genome amplification (WGA) has been performed using modified PCR procedures. These procedures have relied on the nonspecific amplification of the genome using primers that bind under low-stringency conditions to the DNA template. PCR-based approaches differ mainly in terms of the type of primer employed in the reaction: in primer-extension-preamplification (PEP), short 15 base random primers are used in an initial cycling reaction at low stringency to make multiple random copies of segments of the genome. This product is then used as target for the specific PCR reaction. Amplification bias of favorable sequence contexts leading to uneven representation of the genome is the major drawback of this technique. The generation of increasingly shorter fragments during each round of amplification is a further drawback. Another procedure called degenerate oligonucleotide primed-preamplification (DOP-PCR) uses tagged primers and low stringency amplification for the first few cycles of amplification followed by an increase in annealing stringency in later cycles. The tagged primers are characterized by defined sequence tags at the 3′ and 5′ ends and a random sequence in the centre of the primer. Under the later, more stringent conditions, the target DNA fragments generated during the first cycles containing the amplification tag sequences are amplified preferentially without any further shortening of the fragment length. PCR-based WGA typically employs Taq-like polymerases that possess the disadvantage of introducing variations into the amplified DNA due to their relatively low processivity and fidelity which become compounded by the very high number of amplification cycles used in these methods. This may cause problems in downstream applications such as genotyping analysis. These limitations as well as the relatively poor sequence representation of genomic loci inherent to PCR-based WGA can be overcome by an isothermal reaction called multiple displacement amplification (MDA).

The enzyme that is used for MDA comprises a high processivity polymerase with proofreading and strand-displacement activity. The isothermal reaction is performed at 30° without any change in reaction temperature. The reaction starts with the annealing of multiple random primers to the target DNA and elongation of the primers using a DNA polymerase from the *Bacillus subtilis* phage Phi29. Because the polymerase is able to displace DNA strands in a 5′–3′ direction, the polymerase reaction is not stopped when the elongating strands meet downstream DNA strands. The displaced DNA strand serves again as a target for multiple primed elongation reactions so that the DNA template is amplified exponentially in a branched-like manner, yielding high molecular weight DNA with a good representation of the genomic loci. Compared with PCR-based WGA, the error rate is very low. In particular, the mutation rate of repetitive sequence structures is low because of the limited strand-displacement activity of Phi29-polymerase. This permits reliable genotyping of genomic DNA (e.g., SNP analysis, mutation analysis, identity testing, or analysis of case work samples) on different platforms such as real-time PCR or array analysis.
INSTRUMENTATION

The development of the numerous and varied NAT techniques described in this chapter has been facilitated by the evolution of instrumentation that has served to automate these complex procedures. A general description of the major changes in instrumentation is discussed in this section.

The continuous control of the temperature steps necessary to achieve exponential amplification for PCR assays is carried out by fully automated thermocyclers that consist of a heating block in which the temperature can be rapidly cycled. Temperature changes are induced by water, or more recently, by using the Peltier effect. These instruments may be coupled to a fluorometer apparatus if they are used for real-time PCR analysis. In the latter case certain instruments are equipped with a rotor device that is heated and cooled by air instead of a metal block that typically is used as a heating module. In the case of endpoint PCR, PCR products are usually analyzed according to size on agarose or polyacrylamide gels, or by capillary electrophoresis using fluorophore-labeled primers. They may also be analyzed by an array-based approach or other hybridization procedures.

Because no post-PCR processing or label-separation steps are required, real-time PCR assays are simple to perform, making them useful for high-throughput applications. Real-time PCR instruments combine the properties of a thermocycler and a fluorometer to allow determination of PCR products by fluorescence measurement. In each PCR cycle, either one or several fluorescence readouts are taken to monitor the PCR reaction for generation of amplicons, usually at the extension step of the PCR reaction.

Real-time PCR instruments vary with regard to simultaneous sample throughput (32–384 reaction vessels), sample volume (5–100 μL), excitation source, and detector used. These compositions define the suitable range of fluorescent dyes for multiplex real-time PCR as well as size and heating/cooling principle (see above). The excitation source of real-time thermocyclers is either a laser-based system, halogen bulbs, or light-emitting diodes (LED). Optical filters are used to select the wavelength of interest. In most instruments, the emitted light is detected by a charge-coupled device (CCD) that consists of an array of light-sensitive cells. Light projected onto the CCD is converted to an electric charge, resulting in a signal that is proportional to the light intensity.

The versatility of the PCR assay has resulted in the widespread and diverse use of this technique. With the advent of real-time PCR, it has been possible to design high-throughput instrumentation for automated testing. Similarly, the TMA assay has also been automated. Such technology is used by laboratories doing high-throughput, highly regulated testing, typically blood screening for hepatitis C virus (HCV) or human immunodeficiency virus-1 (HIV-1) because automated tests are ideal in a regulated environment where minimum human intervention is required. The use of NAT in a highly regulated environment has resulted in the development of guidances for managing the quality assurance (QA) and quality control (QC) aspects of testing, as well as the validation of systems and assays as described in the following section.

QUALITY ASSURANCE AND QUALITY CONTROL FOR NAT

This section serves as a general guidance for the development of laboratory- and procedure-specific QC and QA procedures for NAT. Aspects such as waste management, management of radioactive material, or working with hazardous material are not covered. NAT is a technology that offers extreme sensitivity with its ability to generate millions of amplicons from as little as a single nucleic acid template, resulting in a detectable signal. The advantages of this technology can be offset by the necessity of establishing complex assay protocols and the requirement to follow carefully very stringent QC/QA protocols. Deviation from these protocols can cause major problems, such as false positive results due to the contamination of templates by amplicons generated in previous assay runs. Similarly, failure to control inhibitors could lead to suboptimal amplification and possible false negative results. Given the myriad factors that can greatly influence the outcome of a NAT assay, all aspects concerning NAT need to be covered by appropriate and stringent QC/QA procedures. This requires careful facility design, workflow, and selection of equipment suitable to the purpose. Data recording, record keeping, and data interpretation are other aspects that should be covered by QC/QA. Thus, QA for NAT assays includes assay validation, establishment of acceptance criteria and specifications, and adherence to good manufacturing/laboratory practices. These aspects are also described in this section. In addition, reference should be made to other published guidances such as the ICH Guideline Validation of Analytical Methods: Methodology (Q2B) and the NCCLS Guidelines.

Laboratory QC/QA

An NAT laboratory should be designed and operated in a manner that prevents contamination of reactions with products from previous amplifications (carry-over) as well as cross-contamination between samples. Historically, the application of PCR required strict separation of the various steps of the assay in order to prevent cross-contamination of PCR by amplicons. This was necessary because early procedures for analysis of PCR products involved the transfer of the product, which potentially could lead to contamination. Therefore, in an open system the best measure to prevent contamination has been the strict separation of working areas for individual process steps. This includes individual areas for template preparation, master mix setup, distribution of the master mix to individual reaction wells and addition of template, space for cycling the PCR assays and, optionally, a separate work space for PCR product analysis. These requirements are not necessary with closed systems. With both open and closed systems it is still necessary to take additional precautions. These safety measures include UV illumination of work spaces overnight to inactivate residual DNA by crosslinking. In case of contamination, laboratory benches and pipettes can be decontaminated by cleaning with a 10% solution of commercial bleach, which usually contains about 5% sodium hypochlorite, taking appropriate safety measures such as wearing gloves and eye protection. Afterwards, benches and pipettes should be rinsed with distilled water. A unidirectional workflow will reduce the opportunity for contamination to occur. Also, no materials, supplies, or equipment should be exchanged between designated working areas or rooms.
Equipment QC/QA

Other good laboratory practices that are related to the prevention of carry-over contamination include the use of suitable and clean equipment. Generally, disposable consumables (tubes, pipette tips, etc.) are highly preferable to reusable equipment. The use of disposable tips containing hydrophobic filters is another very effective measure to minimize cross-contamination. All samples, primer, probes, etc. must be labeled with relevant information such as identity of the content, date of use or preparation, expiration date, concentration, and storage information. Dedicated laboratory coats or disposable lab coats should be available in each room (or section) of the NAT laboratory. Appropriate gloves should be used during all processing steps to prevent sample contamination. The gloves should be changed frequently. Because heat sterilization does not completely destroy DNA, PCR products may lead to detectable contamination of, for example, glass surfaces. Following unique sterilization procedures for different materials such as waste and glass laboratory equipment is advisable.

Carry-Over Prevention with Uracil-N-Glycosylase

Contamination by PCR product carry-over can be mitigated by using the commercially available uracil-N-glycosylase (UNG) procedure. The procedure involves substituting 2′-deoxyuridine 5′-triphosphate (dUTP) for 2′-deoxythymidine 5′-triphosphate (dTTP) in the PCR setup and treating all PCR mixtures with UNG prior to PCR amplification, which can be easily incorporated as a first step into PCR cycling programs. Incorporating dUTP into the amplicon makes the PCR products biochemically distinct from the native DNA template. The enzyme UNG cleaves the deoxyuridine-containing PCR products by opening the deoxyribose ring at the C1 position. When the deoxyuridine-containing DNA is heated during the first thermal cycle, the amplicon DNA chain breaks at the position of the deoxyuridine at the alkaline pH of the PCR reaction mixture and thereby renders the carried-over PCR product nonamplifiable. Thus, any previously generated U-containing amplicon that might have contaminated another sample will become nonamplifiable. As a consequence, false positive results can be avoided. However, it should be noted that UNG has concentration limits above which it does not fully remove PCR carry-over products.

VALIDATION OF NAT SYSTEMS

Assay validation is achieved by
1. ensuring the quality and consistency of assay components, including primers, probes, and enzymes; (including shelf life and contamination control) and
2. establishing the performance characteristics of the NAT assay in terms of reproducibility, accuracy, ruggedness, robustness, specificity, precision, and analytical and clinical sensitivity.

The analytical sensitivity of an assay is defined as the minimum concentration of a reference reagent or standard detected by the test while the clinical sensitivity of a test is determined by testing clinical specimens and determining the 95% LOD. The clinical sensitivity of a test is not necessarily the same as the analytical sensitivity. The closer the reference or standard material is to the samples being tested the closer the correlation.

The principal steps of assay validation are
1. sample preparation;
2. consistent production of critical reagents;
3. use of controls, calibrators, and quantitation standards;
4. specimen and reagent stability;
5. functionality of instruments and software;
6. operator training; and
7. laboratory surveillance for proficiency.

Following assay validation, further QA is necessary to monitor specifications and functional characteristics that have been established by the use of well-characterized reagents of known potency.

Quality Control of Reagents

DNA TEMPLATES

The test specimens used are usually, but not limited to, whole blood, plasma, and serum. Specimen preparation is a key step in the NAT assay and has a major influence on the performance and variability of the assay. Specimen collection is the first step in sample preparation. QC/QA staff should carefully evaluate the effects on the integrity of DNA of collection tubes and temperatures during sample transport. To prevent cross-contamination during specimen collection, aseptic techniques should be used along with closed sampling systems in order to avoid specimen contamination. The use of appropriate sample handling techniques, temperature conditions, and anticoagulants or preservatives should help reduce the risk of contamination. Anticoagulants such as heparin or EDTA may interfere with the NAT assay.

SAMPLE EXTRACTION

The buffers, reagents, and detergent or chaotropic agents used for nucleic acid extraction should be evaluated for inhibitory effects on the NAT assay. Extraction controls, including spiked materials, should be included to monitor the efficiency and reproducibility of the extraction method. Reproducibility of the sample preparation method should be determined under the specimen processing conditions, including sample handling, storage, and shipping conditions. DNA is generally stable, but personnel should take care to avoid storage at refrigerated temperatures for extended periods of time to avoid sample

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degradation. Repeated freeze–thaw cycles can sometimes cause DNA fragmentation. In the case that the target is RNA, it should be noted that RNA is very unstable and specimens should be frozen.

PRIMERS

Primers and probes should be qualified in terms of purity, identity, and functional potency. Purity can be assessed by use of HPLC or mass spectrometry; identity can be established by sequencing; and functionality can be established by the use of reference reagents. However, in many cases, these methods may not be available for in-house testing. In these cases, it may be sufficient to compare lot-to-lot variation of purity and functional potency using relevant methods available in-house coupled with the use of reference reagents.

DNA POLYMERASES

The functionality of enzymes should be determined using reference materials. Enzyme preparations should be tested for other enzymatic activities; for example, exonucleases and DNA- and RNA-dependent polymerase activities and specifications should be established. Lot-to-lot comparison, as well as comparison with the manufacturer’s CoA should also be done. Storage conditions recommended by the manufacturer should be strictly followed, and appropriate controls should be used to monitor the stability of enzymes.

Run Controls

The use of controls affords the operator assurance that the assay has performed within accepted specifications. In PCR testing, several steps in the testing process, as outlined above, should be monitored and verified. Multiple controls or controls that serve multiple purposes may be needed for a PCR assay. Controls should reflect the specific technology under development but should typically allow monitoring of ultracentrifugation, extraction, amplification, hybridization, quantitation, contamination, etc. Controls should be similar to the specimen type whenever feasible, although spiked controls may be acceptable.

A negative control is one that does not contain the target sequence or pathogen that is being tested. It should resemble as closely as possible the sample matrix under testing. Multiple negative controls should be examined, including nontarget sequences and nucleic acid-free controls to monitor for false positives resulting from contamination. Because of the high sensitivity of amplification assays, QC/QA personnel highly recommend that sponsors include control measures for the prevention of contamination events.

A positive control is one that contains the target sequence of interest. It should resemble as closely as possible the specimen matrix being tested and should contain an appropriate and defined amount of target sequences. (e.g., kit control).

Specifications for both positive and negative controls should be provided, as well as validation data supporting the proposed assay cut-off/reporting threshold value or the assay’s limit of detection. The laboratory should define the source of the controls and calibrators and have a plan for their continued renewal. Controls can be infectious or non-infectious. In the latter case, validation of viral inactivation should be provided.

Reagent controls are often referred to as blanks and could include samples that have no target sequence, no enzyme, no primers, etc. These controls provide additional information about problems encountered in PCR assays.

An internal control is added to each specimen to ensure the overall validity of the individual test results. Internal controls are used to verify sample extraction, amplification, and detection.

External Quality Assessment and Proficiency Testing

Quality assessment of the laboratory is achieved by participation in periodic competency assessment and laboratory proficiency programs. The latter should include the testing of reference reagents and well-characterized panels to measure the technical proficiency of operators. Therefore, care should be taken to prevent cross-contamination, to monitor workflow, and to ensure careful specimen and test sample handling. Evaluation of operator proficiency should include participation in competency and quality assessment programs. Each operator in a particular laboratory should participate in such programs and should demonstrate comparable results.

Data Management

Complete and consistent documentation of all activities performed and all data generated is necessary. Such documentation does not only require the maintenance of records of the data generated through sample testing but also information about reagents and equipment calibration and maintenance. Moreover, any alteration in the assay procedure needs to be introduced through a planned change control process and documented in such a way that change can be assessed by an independent party.

〈1128〉 NUCLEIC ACID-BASED TECHNIQUES—MICROARRAY

INTRODUCTION

Microarrays are microscopic spots of DNA (measured in micrometers) arranged in an ordered manner (columns and rows) on a planar surface so that each DNA spot can be uniquely identified to facilitate an accurate analysis of the data. The DNA
spots, also called array elements, are specific DNA molecules of known or unknown sequences and can be of similar or different nucleotide lengths. Samples of these mixtures are placed in fixed locations on the microarray.

Unlike conventional probes, which are a specific DNA or RNA sequence labeled with radioactive, fluorescent, or chemiluminescent tags (see Nucleic Acid-Based Techniques—General (1125), Glossary), the array elements are referred to as probes when the sequence information of the array elements is known, despite not being labeled. In this context, the target refers to labeled nucleic acids in solutions that are hybridized to the array elements or probes. The purpose of a microarray experiment is to identify the sequence of these labeled nucleic acids and/or determine their content. Compendial applications at this time are limited but may increase with wider use of microarrays in diagnostics and in drug discovery, development, registration, and control applications. When used for compendial purposes, standard assay development and validation approaches with availability of suitable reference materials are likely to apply.

Microarrays can range from hundreds to thousands of array elements (low density), to hundreds of thousands of array elements (high density), to millions of array elements (very high density). In addition to the use of planar surfaces for microarrays, the array elements can also be immobilized on individual support particles, such as beads. In these cases the array elements are identified by the particles themselves rather than specific locations on an array. The advantages of using microscopic spots on the array include high density, fast hybridization kinetics, and low sample volumes. Microarrays greatly speed up the acquisition of data, and in some cases increase the predictive power of results, by comparison with conventional nucleic acid-based assays. This is achieved by miniaturization, multiplexing, and parallel execution of nucleic acid-based tests that traditionally are performed in tubes, plates, or capillaries as described in general chapter (1125) (see also Nucleic Acid-Based Techniques—Extraction, Detection, and Sequencing (1126), Nucleic Acid-Based Techniques—Amplification (1127), Nucleic Acid-Based Techniques—Genotyping (1129), and Nucleic Acid-Based Techniques—Approaches for Detecting Trace Nucleic Acids (Residual DNA Testing) (1130)).

The principle of microarray analysis is the specific binding of the target DNA molecules to the probes or array elements. The ordered array of rows and columns of spots allows highly automated detection and analysis. DNA microarrays are manufactured, processed, detected, and analyzed in a number of different ways and have many applications. With the aid of computers, laboratory automation, and high-resolution detection devices, microarrays produce large amounts of data and are the analytical tool of choice to unravel the molecular complexity of DNA or expressed RNA.

The basic principles of nucleic acid amplification technologies (NAT) and definitions of the various techniques are described in chapter (1127). The present chapter covers the general field of microarrays, but detailed treatment of various application-specific microarrays, including data analysis and validation, are excluded from this chapter at this time. The following sections address the major applications of microarrays, sample processing, labeling, workflow, detection, and analysis of data. Several of these sections, for example, sample preparation and labeling, overlap with chapters (1126) and (1127), and cross references are made accordingly. Finally, regulatory aspects of microarrays will be discussed.

### GENERAL PRINCIPLES OF MICROARRAY EXPERIMENTS

#### Types and Applications

Microarrays are most widely used in three types of analysis: gene expression, microarray-based comparative genomic hybridization (or array comparative genome hybridization, aCGH), and single nucleotide polymorphism (SNP). In brief, gene expression microarrays generally measure messenger RNA in a cell; aCGH analyzes DNA copy number variations, chromosomal additions, and deletions in genomic DNA; and SNP microarrays are used in genotyping to analyze single nucleotide polymorphisms (see (1129)). Within each type of microarray, various platforms, both manual and with various levels of automation, are available. Table 1 summarizes the three major types and most common applications, as well as the target for each application, the probe, and the complementary nucleic acid techniques (see (1126), (1127), and (1129)).

<table>
<thead>
<tr>
<th>Types</th>
<th>Application</th>
<th>Target</th>
<th>Probe</th>
<th>Complementary Technology</th>
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<tr>
<td>Gene Expression</td>
<td>Gene Expression</td>
<td>mRNA</td>
<td>Oligonucleotide/cDNA</td>
<td>qRT/PCR, Northern Blotting</td>
</tr>
<tr>
<td>aCGH</td>
<td>aCGH</td>
<td>DNA</td>
<td>Oligonucleotide/ cDNA/Pac, Yac, Bac</td>
<td>Cytogenetic chromosome analysis</td>
</tr>
<tr>
<td>SNP Genotyping</td>
<td>SNP</td>
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<td>Oligonucleotides</td>
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<tr>
<td>SNP</td>
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<td>SNP</td>
<td>Oligonucleotide</td>
<td>Amplicon</td>
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### GENE EXPRESSION MICROARRAYS

Gene expression microarrays are used to measure the relative level at which a certain gene is expressed. They are a powerful tool for target gene discovery, molecular tumor characterization, diagnosis, classification, treatment, and monitoring of diseases. Underlying molecular subgroups that are active in diseases have been identified by observing distinct and recurring gene expression subsets found within diseased tissues. Gene expression microarrays are also used to measure changes in gene expression over a given period of time, e.g., within various stages of a cell cycle or by identification of gene mutation(s) that lead to cancerous growth. Another application for gene expression microarrays is the development of new drugs, e.g. by measuring the down-regulation of a gene associated with a particular disease to monitor the effectiveness of a new drug. When the expression levels from a set of genes are measured, the term gene expression signature (biomarker or classifier) is used.
Other examples of biomarkers or classifiers are drug activity classifiers that are used to diagnose the mechanism of action of a drug or toxicity classifiers that are used to diagnose and develop dosage parameters for a patient.

ACGH MICROARRAYS

In contrast to gene expression microarrays, aCGH microarrays target segments of DNA rather than individual genes (this is, similar to chromosomal banding and traditional comparative genomic hybridization). In an aCGH microarray, the array elements, which are large pieces of genomic DNA or specially designed oligonucleotides, are used to identify a known chromosomal location or changes. The primary advantage of aCGH is the ability to detect DNA copy changes at multiple loci in a single assay and to do so at a much greater resolution compared to traditional CGH. Depending on their design, aCGH microarrays provide distinct advantages over conventional cytogenetic analysis such as karyotyping and fluorescence in situ hybridization (FISH) because they have the potential to detect the majority of microscopic and submicroscopic chromosomal abnormalities. Compared to aCGH, these conventional cytogenetic techniques have low throughput, are labor-intensive, and often require specially trained staff to perform tests in a consistent manner. aCGH microarrays are also useful for the detection of cancer by monitoring the loci of oncogenes and tumor suppressor genes.

SNP MICROARRAYS

SNP microarrays identify the presence of known sequence polymorphisms by analysis of the pattern of hybridization to a series of probes that are specifically complementary either to wild-type or mutant sequences. If the SNP or set of SNPs associated with a particular disease are known, SNP microarrays can be used to identify a disease in an individual. SNP microarrays provide an efficient and inexpensive tool for simultaneously studying multiple genetic variations in multiple samples.

Design of Microarrays

The following sections discuss the design of the three types of microarrays described above and the suitability of the materials used for the microarray probes for each of the three types.

GENE EXPRESSION MICROARRAY

These microarrays are the most common type of microarray in use today. The array elements consist of either cDNA derived from mRNA of known genes but of unknown sequence, or oligonucleotides for which detailed sequence information is available. Oligonucleotides are preferred array elements because of the affordable cost of synthesis and the large amount of sequence information now available for specific genes or gene fragments. These can be arrayed in specific patterns to enable accurate analysis of related gene sequences and gene families in a single hybridization assay. The following general principles apply to oligonucleotide design for gene expression microarrays:

1. Oligonucleotides should be 25–70 mers.
2. Oligonucleotides should include appropriate controls (i.e., oligonucleotides corresponding to sequences from a different organism).
3. All oligonucleotides should map to within 1000 nucleotides of the 3’ end of cDNAs and should correspond to the coding strand.
4. Sequence repeats, stretches of polyA, G, C, and T and extremes of T should be avoided.
5. Oligonucleotides should be compared to sequences in existing databases to avoid cross-reactivity (less than 70% sequence identity with nontarget sequences is preferable).

In addition to oligonucleotides, PCR amplicons and double-stranded DNA (dsDNA) are also used as probes. However, the PCR amplicons require purification to remove enzymes, salts, nucleotides, and other contaminants from the amplification process that could interfere with the binding of the probes and could also inhibit hybridization. In addition, the preparation of dsDNA probes for spotting is labor intensive and expensive. Moreover, dsDNA probes can have repetitive sequences that compromise hybridization specificity. When sequence information is unavailable, dsDNA remain the probes of choice because unknown dsDNA probes can still be used to study gene expression.

ACGH MICROARRAYS

These microarrays traditionally use bacterial artificial chromosomes (BACs) of 100–200 kilo-base pairs per DNA segment as the array elements. However, the large-scale DNA isolations or PCR amplifications of such large-insert clones are elaborate and time consuming. As is the case in expression profiling applications, aCGH microarrays have transitioned from dsDNA targets to oligonucleotide targets. Oligonucleotide libraries or ready-made microarrays can now be purchased, saving considerable time and effort.

SNP MICROARRAYS

Depending on the application, SNP microarrays can use both amplicons and oligonucleotides as probes. In one of the most common formats to detect mutations in a gene sequence, the probe is that of a single gene in which the sequence differs by a single nucleotide polymorphism from the sequence of the other probes for that gene in the same microarray. For the discrimination of only one mismatch, short oligonucleotide probes (15–30 bp) maximize the destabilization caused by mispairing and are therefore used for the detection of SNPs.
Manufacturing of Microarrays

Microarray elements are deposited onto a solid support, the most widely used of which is glass. Microarray manufacturing can be divided into two main categories, direct synthesis of the probes on the microarray (in situ) or synthesis of the probes before spotting on the microarray (ex situ). In situ synthesis is generally used for higher density microarrays but is limited to nucleotides of approximately 25–100 bases. With increasing nucleotide length, the likelihood of truncated products increases because of the limited stability of building oligonucleotides in situ. In contrast, ex situ microarray manufacturing can put any premade material into a microarray format, including oligonucleotides, PCR products (amplicons), complementary DNA (cDNAs), and BACs.

The main techniques for in situ synthesis are photolithography, maskless lithography, and ink jetting. Microarrays are generally manufactured commercially, although for a small number of low-density microarrays, the end user can manufacture the microarrays using a low-throughput microarray manufacturing robotic instrument (a personal microarrayer). However, only maskless lithography and ink jetting are available for end user manufacturing. In photolithography, a glass substrate containing a photomask, which is chemically prepared so that particular nucleotides bind to specific positions, is used to synthesize the oligonucleotides on the substrate. The masks predetermine which of the nucleotides are activated when flooded with one of the four types of nucleotides. The process is repeated until the required number of bases is synthesized. The manufacture of these microarrays uses computer algorithms and multiple spots to cover the gene of interest. Maskless lithography uses a digital micromirror device that uses a solid-state array of miniature aluminum mirrors to create virtual masks that replace the physical photomasks. A computer controls the desired pattern of UV light via individual mirrors. Each digital micromirror in turn controls the pattern of UV light projected onto the glass in the reaction chamber, which is coupled to a DNA synthesizer. The UV light selectively cleaves a UV-labile protecting group at the precise location where the next nucleotide will be coupled. The patterns are coordinated with the DNA synthesis chemistry in a parallel, combinatorial manner so that hundreds of thousands of unique oligonucleotides can be synthesized in a single microarray. Ink jetting is accomplished by building up the nucleotides, base-by-base, in repetitive print layers using standard phosphoramidite chemistry. Inkjet heads similar to those used in commercial inkjet printers are connected to bottles that contain the four different phosphoramidite nucleotides that make up the building blocks of in situ nucleic acid synthesis. The advantages of inkjetting and maskless lithography are flexibility in design and the ability to make small batches of arrays quickly.

The two main types of ex situ manufacturing techniques are microspotting pins (contact printing) and piezoelectric printing (noncontact). The technology excels at printing multiple probes many times over numerous surfaces with one small-volume loading of probe. Spot size and delivery volume are controlled by the size of the end of the tip, and many tip sizes are available. A piezoelectric printing mechanism uses a small dielectric crystal in contact with a glass capillary that holds the sample fluid. Application of the voltage results in ejection of fluid from the tip, resulting in drop volumes from hundreds of picoliters to several microliters.

General Experimental Considerations

Regardless of the type and application, all microarray experiments have a similar workflow: amplification step, labeling, hybridization, and wash steps, followed by scanning, quantitation, and reporting. The experimental design determines the type of microarray used, number of spots required, and the specific sets of nucleic acids on the microarray. The experimental design also influences the platform used, such as the number of spots, surface type, nucleic acid type, throughput, resolution, and number of colors that can be detected in a single assay. Platforms can be open (support is available from multiple vendors) or closed (support from a single vendor). In general, experimental designs that require a high density of spots and quantitation are more difficult and expensive to implement than qualitative assays.

Microarray Sample Considerations

Sample extraction, isolation, and preparation should be carefully chosen in order not to alter the ability of the resulting target to hybridize to the microarray. In general, sample preparation issues are the same for microarrays as for other laboratory techniques such as qPCR (quantitative PCR) and sequencing (described in chapters (1126) and (1127)). RNA, cDNA, genomic DNA, and PCR products are some of the sample types analyzed with microarrays. In some genotyping applications, specific alleles are used both as array elements and targets.

As with any nucleic acid technique, the quality of the nucleic acid is critical for the microarray experiment. The nucleic acid should be pure, intact, and accurately quantitated before use (1126). In particular, the presence of contaminating DNA in total RNA samples may cause problems in microarray analysis because some labeling methods label both RNA and DNA with equal efficiency. For some applications in which even trace contaminants with either RNA or DNA may interfere, pretreatment with DNase or RNase may be necessary. For example, contaminating, labeled DNA can hybridize with microarray targets leading to high-level hybridization signals that are not derived from RNA transcripts, thus resulting in an inaccurate estimation of the target RNA concentration because both nucleic acid species are quantitated at the same wavelength.

A major consideration in any microarray experiment is the availability of adequate amounts of sample nucleic acid for analysis. For example, sample from laser-capture microdissection, needle tissue biopsies, or other small clinical samples do not yield sufficient RNA (for expression microarrays) or DNA (for aCGH microarrays) and must be amplified before analysis. It is critical that the amplification procedures for amplification of mRNA be so designed that the final mixture of amplicons accurately reflect the distribution of mRNA species in the sample. Uniform amplification of genomic DNA for aCGH microarrays can be achieved by the use of multiple displacement amplification (MDA), which overcomes the nonuniform amplification of genomic DNA that occurs in PCR-based amplification methods that use degenerate oligonucleotide primed PCR (DOP-PCR). For SNP arrays where specific alleles are the target of interest, nonuniform amplification is not an issue, and samples can be amplified (and labeled) by PCR, multiplex PCR, and WGA (see (1127)).
Microarray Labeling

The targets for a microarray are a population of nucleic acids that are extracted from a sample and are appropriately labeled. Many methods can be used for labeling targets (see (1127)), but fluorescent labeling is the most widely used because it offers high sensitivity and a superior dynamic range. An added advantage is the ability to detect two or more signals in a single experiment. The method of labeling depends on the microarray type. The two methods used to fluorescently label targets for gene expression microarrays, direct and indirect labeling, have been described in (1127). In general, the second method (indirect labeling), in which the label is added via a linker, requires less starting material and is less expensive. Published reports have shown that this method yields results similar to those obtained from directly labeled samples. In microarray aCGH, a patient’s DNA and reference DNA (300–1000 ng) are typically fluorescently labeled with red and green fluorescent dyes, respectively, often using a random priming protocol. Random prime labeling uses a high concentration of Klenow enzyme whereby genomic DNA is digested with restriction enzymes and hybridized with random primers. The primers are extended by the 3’–5’ polymerase activity of Klenow, resulting in a strand displacement activity with the direct incorporation of labeled nucleotides. SNP microarrays using oligonucleotides as array elements are labeled using fluorescently labeled nucleotides in both single and multiplexed PCR reactions, followed by a purification step to remove unincorporated dyes. Where amplicons are used as array element, labeled oligonucleotide probes are synthesized using phosphoramidite chemistry.

Hybridization and Wash

Hybridization should be carried out under conditions that minimize annealing of noncomplementary fragments. The wash steps following a hybridization reaction are optimized to provide the highest possible specificity, signal-to-noise ratio, and reproducibility (see (1126)). Before hybridization, double-stranded probes and targets should be denatured, and nonspecific sites should be blocked. Microarray surface chemistries are designed to capture all nucleic acids with high efficiency, so the free-binding groups on the surface must be blocked or inactivated to prevent nonspecific binding of labeled material that could compromise the signal-to-noise ratio. Surfaces are blocked and washed with various aqueous-based buffers that typically include salts, detergents, and blocking agents such as low molecular weight, hydrolyzed proteins. The purpose of the posthybridization washes is to remove all unattached and nonspecifically bound label from the surface and probes. In general, both automated and manual washes are done in saline sodium citrate/sodium dodecyl sulfate (SSC/SDS) buffers of various concentrations and at different elevated temperatures depending on the stringency required. After the final wash step, microarrays using fluorescent targets are dried immediately by centrifugation or in a nitrogen stream. Hybridized microarrays must be stored in the dark and should be scanned as soon as possible. Some fluorescent dyes used in microarray analysis are subject to degradation by environmental ozone, and in these cases ozone levels in the experimental environment must be less than 5 parts per billion. Specialized ozone-free hoods are made to protect microarray dyes.

Microarray Detection

Regardless of the microarray type, each spot on a microarray represents a unique probe sequence to which a single, labeled target is bound, and this specific binding allows detection and quantitation of the target. This is achieved by the emission of light (photons) at a particular wavelength by the fluorescently labeled duplexes when the microarray is exposed to light of specific wavelength from an excitation source. The emitted fluorescent light is converted to electrical energy by a detector. The detector is either a photomultiplier tube (PMT) or a charge-coupled device (CCD) with specially designed optical paths that collect the raw data from microarrays (scanning). The detector filters and optical paths are designed to detect specific fluorescent dyes at sufficient resolution while eliminating crosstalk when two or more dyes are used on a single microarray. The resulting signal is proportional to the number of photons emitted by the microarray. These signals are used to create a digitized image showing the presence and quantitation of specific targets.

Samples can be scanned from a single wavelength channel or can be sequentially scanned from two channels. For instance, for a single-channel microarray platform, a sample is typically labeled with a fluorophore that emits a signal in the red channel. For a dual-channel microarray format, a second sample can be labeled with a dye that emits in the green channel. Dual labeling is used in some experimental designs, such as expression microarrays, to measure the overexpression of a gene associated with a disease state. In such experiments, cDNAs derived from the mRNA of normal and diseased tissues are differentially labeled, mixed, and tested on the same slide in a competitive hybridization reaction. The resulting ratios of the two colors reflect the relative abundance of the labeled material within each sample. Similarly, calculating the fluorescent ratios from each target on an aCGH microarray allows the mapping of gains and losses for a chromosome of interest.

Microarray Image Processing

Most microarray scanners detect and acquire one, two, or more colors (via one, two, or more channels). The optical path of the system minimizes overlap between the spectra (crosstalk) and allows acquisition of two spectrally separate images. In many cases, the images are represented as a red and a green image. When two colors are used, the ratio of the two fluorescence images eliminates artifacts caused by regional bias and irregular spot size. When one color is used, the fluorescence signals from two or more microarrays are normalized and can be compared with each other. Diameters of spots printed on the arrays range from 10 μm to just under 1000 μm, and the resolution of scanners ranges from 1 to 50 μm. Thus, depending on scanner resolution, variable amounts of pixel data can be collected per scan over an entire microarray.
MICROARRAY IMAGE ANALYSIS

The analysis of scanned images usually involves three tasks: spot finding or gridding, image segmentation, and spot quantification.

Spots are initially assigned specific coordinates, and the process of spot finding or gridding can range from manual to fully automatic, depending on the image-processing software used. This takes into account the individual size and shape of each spot and adjusts for uneven rows and columns that may be produced by the printing process.

The process of segmentation partitions the entire image to foreground or background pixels and relies on the spatial and intensity properties of each pixel. There are four main types of signal segmentation that have been used for spotted arrays. The simplest method is spatial segmentation which places two circles (inner and outer circles) of fixed but different sizes over each spot to demarcate probe signal from the immediate background signal. On the one hand, because of the irregularity of spot sizes on some microarrays, the actual area inner circle may be larger than the diameter of a spot and thus will contain background pixels. On the other hand, artifacts and signal can be found in the area between the inner and outer circles and contribute to the background signal. The second method, intensity-based segmentation, distinguishes signal pixels from background pixels based on the spot intensities within a target region. In this case, a certain percentage of pixels within the top-ranked intensities may be classified as signal pixels. The advantages of this method are simplicity and speed, but the drawback is the inability to distinguish between artifacts and signal and the tendency to detect low signals that are close to background. The third method is a statistical approach known as Mann-Whitney segmentation that combines information from spatial and intensity-based analysis. Here, background pixels located outside the inner circle set are used to determine a threshold intensity level for a signal within the inner circle. The limitation of this method is that a large amount of spot irregularities and artifacts can reduce its accuracy. The fourth method, the trimmed measurement segmentation method, also combines spatial and intensity information and measures signal distributions inside and outside the inner circle. The method trims the upper and lower extremes of each distribution to allow removal of signal from artifacts and incorrectly located background or foreground signal pixels.

The main assumption of spot quantification is that the total fluorescent intensity from a spot is proportional to the expression level of the labeled transcript. This is highly dependent on a number of factors, including target preparation, hybridization conditions, and signal detection within the linear dynamic range. If the amount of probe deposited during the microarray manufacturing procedure varies from spot to spot and from array to array, thus resulting in different sized spots, the sum or total signal intensity can be variable and inaccurate. To correct for this variation, microarrays should be spotted via homogenous surface chemistry that has a fixed binding capacity. This ensures the same amount of probe at each spot location. Alternatively, spots can be quantified by taking the mean, median, or mode of intensities of all signal pixels determined to be foreground signal. The more robust methods that protect against outlier signals are the trimmed mean (where a certain percentage of top and bottom signals are trimmed before calculation of the mean) and median signal intensities. When two different fluorophores are used, the intensity ratio can be used to correct for variable probe amounts and can be calculated from mean, median, and mode intensities from each channel.

MICROARRAY DATA ANALYSIS

Particularly dense formats of microarrays that contain tens of thousands to millions of probes per chip or slide generate a large volume of raw data per array, which requires the use of specialized data-analysis software. Microarray software programs are designed to extract primary data, normalize the data to remove the influence of experimental variation, and link probes to relevant gene and sequence-derived targets. Software programs are also available to apply statistical methods, analyze, visually display, and manage data in order to extract biologically meaningful information. The major parts of data analysis are normalization, background correction, and ratio calculation.

Normalization systematically adjusts microarray raw data in an effort to reduce the variability brought about by differences in the manufacture and processing of the microarrays and by technical variables so that true biological differences between samples can be detected. The wide range of normalization methods precludes a detailed discussion of the topic in this chapter, and currently there are no standards for normalization. Commonly used algorithms are selected based on the microarray type, the number of fluorophores used, and the samples being studied. Some methods are built into the manufacturer’s software, but others are available from commercial sources or open-source software providers.

Background correction eliminates low levels of noise in microarrays stemming from both the inherent noise of the detection instruments and from the surface chemistry used in manufacturing. Several contaminants acquired from microarray processing can cause high levels of background that must be corrected before data analysis.

In two-color microarrays, the ratio of signal intensities of array elements of two co-hybridized samples is used as a relative measure of gene expression. In single-channel systems, the ratio can be calculated between signals taken from two different samples (one sample is a reference sample) hybridized on individual microarrays. Thus, the resulting data from microarrays does not represent an absolute quantification but rather a relative level of RNA or DNA against a reference sample or control.

Quality Control and Quality Assurance

As with any diagnostic assay, quality control and quality assurance are critically important. Microarrays must demonstrate robustness and reproducibility. The general quality control and assurance steps outlined in chapter 1127 for nucleic acids and NAT also apply to microarrays. Unlike other diagnostic tests, no reference reagents are available at present for quality control of microarrays, and regulatory guidance is emerging. FDA has issued a draft guideline titled “In Vitro Diagnostic Multivariate Index Assays.” This guidance addresses the definition and regulatory status of a class of in vitro diagnostic devices referred to as in vitro diagnostic multivariate index assays (IVDMIAs), and microarrays fall into this category. The guidance addresses premarket pathways and postmarket requirements with respect to IVDMIAs.
Several unintended sources of variability that are specific to microarrays can extensively affect signal intensities and the accurate derivation of a true signal that accurately reflects the labeled transcript. A major source of variability is spot quality. Measurements of spot quality at the processing stage permit removal of spots with poor or questionable quality. Other sources of variability are artifacts, for example, regional shifts (rise or fall) in an array’s overall signal that can be visualized within single chips or in-composite data derived from multiple chips. These changes can be distinguished from actual variability because they are nonrandom, and patterns can be detected by visualizing signals over the entire area of the chip. When dyes of different spectral properties are used to label two different samples in a competitive hybridization reaction using a single array, differences may arise because of labeling bias rather than gene expression level. For instance, the green channel may appear consistently brighter than the red channel despite the fact that there are no real differences in expression. Hybridization with reverse dyes can ensure detection and elimination of dye bias effects. As with any quantitative assay for RNA, the integrity of the sample affects its measurement, and sample quality is an important determinant for accuracy. For instance, because labeling is directed from the 3’ to the 5’ end but RNA degrades from the 5’ end, degraded RNA leads to high 3’/5’ ratios, resulting in nonuniform labeling across the entire transcript. Finally, variability can be introduced during the processing of microarrays, which is a relatively complex procedure that involves multiple steps such as labeling, hybridization, washing, and staining (technical variables). Such variability can mask true differences in the samples tested.

When used as a diagnostic test, the microarray should demonstrate robustness, reproducibility, a high degree of correlation to the original format, and reliable prognosis prediction. The microarray ideally should contain at least 2–3 replicate spots for each reporter gene to ensure intra-assay reproducibility. With a two-channel microarray, a reference sample pool can be hybridized in the complimentary fluorescent channel so that data can be expressed as log ratios, which reduces the need for extensive normalization. Interassay reproducibility of test results and stability over time can be tracked by using a number of reference samples that, when labeled and hybridized, represent a spectrum of predictive endpoints (for instance, high risk, borderline risk, low risk) and that should fall within a predetermined range of results. Failure of these controls should result in rejection of results of samples in the same assay run. If the assay is performed at many sites, site-to-site reproducibility is imperative and must be assessed. The reproducibility of the assay with regard to tissue extraction also must be determined, and the quality of tissue specimens or RNA should be specified clearly (for instance, percentage of tumor cells within a specimen).

In conclusion, microarray experiments should be carefully designed and conducted in order to minimize variability and to yield data that accurately relate to the samples analyzed. In addition, biomarkers of interest should be analyzed and verified using an alternative platform such as qRT-PCR that should be shown to be reproducibly detected in the same and different samples. The development of reference standards, especially when microarrays are used as diagnostic tests, is the next step to ensuring the quality and validation of microarray results. With the shift from custom-built to commercial microarrays, issues with reproducibility, standardization, and quality control have been largely addressed by the stringent quality controls used in commercial manufacturing.

**INTRODUCTION**

This chapter outlines techniques for detecting single-base DNA differences and other types of polymorphic DNA sequences that occur in the three billion bases that make up the human genome. The most common genetic variation is a single nucleotide polymorphism (SNP), which is a simple change in one base of the gene sequence. SNPs occur on average every 1000 bases and account for a significant amount of inter-individual variability. SNPs can predispose individuals to disease or influence their response to a drug. Approximately 1.8 million human SNP loci have been identified, and more are likely to be discovered in the coming years.

Common approaches for detecting SNPs and other types of polymorphic DNA sequences are described in the following sections. These approaches encompass a variety of techniques, such as nucleic acid amplification techniques (NAT), real-time NAT, and microarrays, the principles of which are covered in more detail in related chapters. This chapter focuses on the specific modifications of the techniques that are necessary to enable detection of single base differences.

**SNP GENOTYPING TECHNOLOGIES**

Although the usefulness of studying SNPs for gene mapping and disease association studies is apparent, a single standardized procedure for SNP genotyping has not been adopted. Various approaches for performing SNP genotyping have been developed to meet a wide range of needs, including throughput capacity, ease of assay design, accuracy, and reliability. Available procedures can also be divided according to whether they are based on identifying known SNPs or whether they can be used to screen for unknown SNPs. To identify the most appropriate SNP genotyping procedure for a specific application, the throughput requirements in terms of the number of SNPs to be analyzed per sample (multiplexing level) and the sample throughput need to be determined because different approaches may work best depending on these requirements.

Most procedures used for genotyping SNPs depend on polymerase chain reaction (PCR) amplification of the genomic regions that span the SNPs followed by the actual genotyping reaction. PCR provides the required sensitivity and specificity for distinguishing between heterozygous and homozygous genotypes in large, complex genomes. The difficulty of designing and carrying out multiplex PCR reactions limits the throughput of many of the current SNP genotyping assays. The following sections outline several of the major approaches currently in use for SNP genotyping. In many cases the underlying technology can be modified to meet the specific application requirements in terms of sample throughput and number of SNPs detected. In general,

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real-time PCR-based procedures are better suited to higher sample numbers, and array-based procedures are better suited to the simultaneous detection of many SNPs. Newer technologies based on multiplexed array formats are also emerging and will be suitable for high sample numbers and many SNP applications.

**Sequencing**

Sequencing is the definitive procedure for DNA analysis, and its use for SNP detection allows unambiguous identification of base changes (see Nucleic Acid-Based Techniques—Extraction, Detection, and Sequencing (1126) for nucleic acid sequencing). The standard technology is expensive, and the procedure is time consuming and labor intensive and suffers from low sample throughput. Sequencing is a useful confirmatory tool, and it has applications in situations when other technologies are not appropriate, but is not the most cost-effective solution for the majority of SNP genotyping applications that require the identification of only one or a few bases.

**Restriction Fragment Length Polymorphism Analysis**

The first widely used procedure for the detection of polymorphisms exploited alterations in restriction enzyme sites caused by SNPs, leading to the gain or loss of cutting events. PCR–restriction fragment length polymorphism (RFLP) analysis comprises PCR amplification of a fragment of interest and subsequent digestion with a restriction enzyme. The fragments produced are typically analyzed by a size fractionation procedure, usually gel electrophoresis. Because of its simplicity, the procedure has been and still is extensively used, although it entails certain limitations: only a subset of polymorphisms that reside in an exon and flank a restriction enzyme site are detectable. This approach can be used only for systems that do not rely on hydrolysis of the probe to generate a signal and is therefore not suitable for hydrolysis probe assays.

**Probe Hybridization**

The basis of many SNP genotyping procedures are DNA hybridizations that make use of the stronger binding of a DNA probe to a perfectly matched complementary target than to a target that contains a single base mismatch. The ability of hybridization with allele-specific oligonucleotides (ASO) to detect a single base mismatch was first shown in the late 1970s and subsequently was used to detect the sickle-cell mutation in the beta-globin gene by Southern blot hybridization. The invention of PCR facilitated the further development of probe-based assays for genotyping SNPs in complex genomes.

The thermal stability of a hybrid between an ASO probe and its SNP-containing target sequence is not only determined by the stringency of the reaction conditions but also by the secondary structure of the target sequence and the nucleotide sequence flanking the SNP. Therefore, prediction a priori of the reaction conditions or the sequence of the ASO probe that will allow optimal discrimination between two alleles using ASO hybridization is difficult. These parameters should be established empirically and separately for each SNP. Consequently, there is no single set of reaction conditions that would be optimal for genotyping all SNPs, which makes the design of multiplex assays based on hybridization with ASO probes an extremely difficult task.

One approach to counter the problem of assay design is to carry out multiplex ASO hybridization reactions on arrays that carry multiple probes for each SNP that will be analyzed. This involves using probe sets in which the SNP occurs at different positions along the probes. It becomes feasible to include large numbers of ASO probes per SNP when one uses high-density arrays that can carry as many as 10^6 probes per cm^2.

Another approach is to use base analogues such as locked nucleic acid (LNA), which is described in detail in Nucleic Acid-Based Techniques—Amplification (1127). For applications that involve few SNPs but many samples, homogeneous real-time PCR approaches have been developed. These include the use of fluorescent probe chemistries such as hydrolysis probes, stem-loop probes, and FRET (fluorescence resonance energy transfer) hybridization probes. The principle of these assays is discussed in more detail in Nucleic Acid-Based Techniques—Amplification (1127). For SNP detection, the basis of many assays is the selective binding of the ASO probe to its perfectly matched target sequence, resulting in energy transfer and generation of a fluorescence signal. Probes designed with specific secondary structures tend to form a stem–loop structure that destabilizes mismatched hybrids, increasing their power of allele distinction as compared with that of linear ASO probes. Hydrolysis probes modified with minor groove-binder molecules that increase target affinity show improved powers of allele discrimination. The use of two probes, each labeled with a different reporter fluorophore, allows both SNP alleles to be detected in a single tube. Limited multiplexing can be achieved by using probes labeled with different fluorophores. In the fluorescent probe–based assays, the increase in fluorescence due to accumulating PCR product is usually monitored in real time in 96-well or 384-well microtiter plates. Alternatively, the fluorescence generated from the two alleles can be measured after completion of the PCR. In this case the results are expressed as a signal ratio that reflects the hybridization of the two oligonucleotides to the target sequence, and so differences in amplification efficiency between samples do not affect interpretation of the genotyping results.

A third approach involves heating the reaction after PCR has been completed in order to dissociate the probe from the target. Each duplex has its own specific Tm, which is defined as the temperature at which 50% of the DNA becomes single stranded. The Tm depends on the stability of the probe–target duplex. Perfectly matched probe–target duplexes have a greater stability and hence a higher Tm than does the same duplex containing a single base mismatch. By continuously monitoring the fluorescence during the heating phase, analysts generate a “melt curve” that measures the changes in fluorescence that result when the probe denatures, or “melts,” away from the amplicon. This approach can be used only for systems that do not rely on hydrolysis of the probe to generate a signal and is therefore not suitable for hydrolysis probe assays.

Because no post-PCR processing or label-separation steps are required, homogeneous real-time PCR assays are simple to perform, making them useful for high-throughput genotyping applications. The optimal probes must be designed individually for each SNP, and the assays are therefore most efficient when a limited number of SNPs is analyzed. The cost of probes modified with fluorescent and quenching moieties may also be a limiting factor in the high-throughput application of the assays.
Primer Extension

In this technique, an oligonucleotide is used to prime DNA synthesis by a polymerase enzyme, as performed in a standard PCR or sequencing reaction. Variations of the technique exist. Allele-specific PCR uses two primers, each fully complementary to one of the SNP alleles, with the SNP position being at the 3' end of the primer, and with a common reverse PCR primer to selectively amplify the SNP alleles. Because only perfectly matched oligonucleotides will prime DNA polymerase extension, product will be detected only from the reaction containing the perfectly matched primer.

Agarose gel electrophoresis is used to detect the amplified products, although homogeneous, real-time, allele-specific PCR approaches have also been developed using primers labeled with different fluorophores or a fluorescent dye that intercalates with the double-stranded PCR products or by performing amplicon detection using probes such as hydrolysis and hairpin (stem-loop) probes. When using intercalating dyes or labeled allele-specific PCR primers without a consecutive target-specific detection reaction or size-separation step, one may find that the specificity of the procedure may be compromised owing to primer-dimers and other spurious amplification products that will not be distinguished from the actual PCR product. A limitation of all variants of allele-specific PCR is that the reaction conditions or primer design for selective allele amplification must be optimized empirically for each SNP. Like the hydrolysis and hairpin probe assays, the homogeneous allele-specific PCR procedures are best suited for the analysis of a limited number of SNPs in large sample collections. Array-based approaches for greater SNP multiplexing have also been developed.

In procedures based on single nucleotide primer extension (sometimes known as minisequencing), allele discrimination is based on the high accuracy of nucleotide incorporation by DNA polymerase. A primer is used, and its 3' end is positioned on the base just preceding the SNP to be tested. The DNA polymerase is then used to incorporate labeled ddNTPs, each labeled with different fluorescent dyes. After the labeled oligonucleotides are separated from the nonincorporated ddNTPs, the results can be scored on a fluorescence plate reader. In addition to fluorescent tags, ddNTPs may be labeled with biotin or hapten aand then detected indirectly through antibodies conjugated to alkaline phosphatase or peroxidase using colorimetric or chemiluminescent markers in ELISA formats.

Multiplexing of this procedure has also been described to reduce costs and improve throughput. In these procedures, the different loci genotyped simultaneously are separated either by gel electrophoresis or by hybridization to arrayed tags. Primer extension directly on a solid support such as a microarray is also possible. The immobilization of the single-stranded primers on the solid support may be through biotin–avidin–streptavidin reaction or covalently via 5' disulfide groups.

Mass spectrometry using techniques such as matrix-assisted laser desorption–ionization time-of-flight mass spectrometry (MALDI-TOF MS) can also be used to determine the identity of the ddNTP incorporated based on mass. A difficulty with MALDI-TOF MS is that the primer extension products must be rigorously purified before measurement to avoid background from biological material present in the sample. Such enzyme-assisted procedures have proven to be more robust and to provide more specific allele discrimination than does ASO hybridization at similar reaction conditions. These features are advantageous for high-throughput applications because the effort required for assay design and optimization is minimized.

Ligation

In the oligonucleotide ligation assay (OLA), oligonucleotides are designed so that they meet at the position of the SNP to be tested. Enzymatic joining, using a DNA ligase, occurs only when the match is perfect. The test is usually performed by designing two oligonucleotides specific for each allele and labeled differently on one side of the SNP, and one common oligonucleotide on the other. Detection of the alleles can be performed directly in the microplate wells by colorimetric approaches. Multiplexing and the use of gel separation have also been described.

OLA has also been used in microarray formats with one of the ligation probes immobilized or with immobilized single stem–loop probes. Alternatively, ligation can be carried out in solution followed by capture of the ligation products on microarrays or on microparticles that carry a generic set of oligonucleotides that are complementary to a "tag" sequence on one of the ligation probes. In practice, thermostable ligases are frequently used for genotyping SNPs in combination with PCR before allele-specific ligase detection reactions. Because the reaction mechanisms for PCR and ligation are different, the reagents for both reactions can be combined. This feature is used in a homogeneous, real-time PCR assay with ligase-mediated genotyping and detection by FRET. Compared with DNA-polymerase-assisted primer extension procedures, a drawback of the OLAs is that detection of each SNP requires three oligonucleotides, which increases the costs of these assays.

Padlock probes are linear oligonucleotides, the ends of which are complementary to the target and have a central stretch of random sequence. When perfectly hybridized to their target sequence, padlock probes can be circularized by ligation, whereas a mismatch with the target sequence prevents ligation. Circularized oligonucleotides can act as templates for DNA-polymerase-assisted rolling circle amplification (RCA). RCA can be used to amplify the ligated circularized padlock probes to a level required for detecting single-copy sequences. A homogeneous, isothermal assay for genotyping individual SNPs in a microtiter plate format has been devised by combining exponential amplification of ligated padlock probes using a branched rolling circle amplification reaction with detection by energy-transfer-labeled hairpin primers.

Displacement

The invader assay uses the property of flap endonucleases (FENs) for removing redundant portions (flap) from the 5' end of a downstream DNA fragment overlapping an upstream (invader) DNA fragment. An invader oligonucleotide is designed with its 3' end on the SNP to be tested. Two oligonucleotide signal probes are also designed, overlapping the polymorphic site and each corresponding to one of the alleles. After displacement of the signal probes by the invader probe, FEN-mediated cleavage occurs only for the perfectly matched allele-specific signal probe. Generation of the cleaved fragment is monitored by using it in a second reaction as an invader probe to cleave a FRET probe. This assay does not require PCR amplification of the locus to be tested, and scoring can be done using a simple fluorescence plate reader.
Pyrosequencing

In the pyrosequencing procedure, primer extension is monitored by enzyme-mediated luminometric detection of pyrophosphate (PPi), which is released on incorporation of deoxynucleotide triphosphates. The genotype of an SNP is deduced by sequential addition and degradation of the four nucleotides using an enzyme in a dedicated instrument that operates in a 96-well or 384-well microtiter plate format. Using pyrosequencing, the apparatus can determine short 30 to 50 bp sequences of DNA that flank an SNP. A limitation of the procedure is that the sequential identification of bases prevents genotyping of several SNPs per reaction in diploid genomes. An advantage of the procedure is that any new polymorphism will be detected. However, specific equipment is needed for the injection of the nucleotides.

Single-Strand Conformation Polymorphism and Heteroduplex Analysis

Single-strand conformation polymorphism (SSCP) and heteroduplex analysis were among the first procedures established for the detection of SNPs. Conventional SSCP analysis involves denaturing PCR-amplified fragments and subsequent formation of sequence-specific secondary and tertiary structural differences between the single strands in nondenaturing gel electrophoresis. The electrophoretic mobility then depends on the 3-D shape of the single-stranded molecules. One single base difference in DNA fragments of up to 300 bp will usually change the conformation in a way that can be detected by nondenaturing PAGE.

The traditional polyacrylamide gels and γP-labeled fragments are frequently being replaced by fluorescently labeled fragments and automated capillary electrophoresis. The simplicity of the procedure, combined with automation and short analysis time, contribute to high-throughput analysis at relatively low cost. If the denatured PCR products are allowed to slowly re-nature, they form DNA duplexes. The duplexes with the same sequence on both strands (homoduplexes) or with a single base pair mismatch on one strand (heteroduplexes) have different electrophoretic mobility in a native gel. In the case of a single base pair substitution, the heteroduplex can easily be separated from a homoduplex.

In other versions of the technique, denaturing high-performance liquid chromatography (DHPLC) is used for the separation of the heteroduplex and homoduplex strands. The mutation analysis with DHPLC can be almost totally automated with an autosampler on one end and a fraction collector on the other. Analysis is rapid (about 5 minutes per sample), and simple evaluation of data distinguishes between single and multiple peaks in the elution profiles, allowing lengths as large as 1.5 kb of DNA to be analyzed. A disadvantage may be the recommended use of Pfu DNA polymerase, which, as a high-fidelity enzyme, allows sharper peaks but may be less successful in amplifying some regions.

Short Tandem Repeat Profiling

A short tandem repeat (STR) is a type of DNA polymorphism that occurs when a pattern of two or more nucleotides is repeated and the repeated sequences are directly adjacent to each other. The pattern can range in length from 2 to 10 bp (e.g., CATG, in a genomic region) and is typically in the noncoding intronic, or upstream/downstream regions. By examining several STR loci and counting how many repeats of a specific STR sequence there are at a given locus, one can create a unique genetic profile of an individual. Currently more than 10,000 STR sequences in the human genome have been published. STR analysis has become the prevalent analysis procedure for determining genetic profiles in forensic cases. STR analysis in the field of forensics came into popularity in the mid to late 1990s. The STRs in use for forensic analysis are tetra- or penta-nucleotide repeats (4 or 5 repeat units) because these give a high degree of error-free data while being robust enough to survive degradation in nonideal conditions. Shorter repeat sequences tend to suffer from artifacts such as stutter and preferential amplification; several genetic diseases are associated with tri-nucleotide repeats, including Huntington’s disease. Longer repeat sequences suffer more highly from environmental degradation and do not amplify by PCR as well as do shorter sequences.

The analysis is performed by extracting nuclear DNA from the cells of a forensic sample of interest and then PCR amplifying specific polymorphic regions of the extracted sample. Once these sequences have been amplified, they are resolved either by gel electrophoresis or capillary electrophoresis, which allow the analyst to enumerate the repeats of the STR sequence in question. If the DNA is resolved by gel electrophoresis, the DNA can be visualized either by silver staining or an intercalating dye such as ethidium bromide or, as in most modern forensics labs, by fluorescent dyes. Instruments built to resolve STR fragments by capillary electrophoresis also use fluorescent dyes. In the United States, 13 core STR loci have been selected as the basis by which an individual genetic profile can be generated. These profiles are stored in local, state, and national DNA databanks such as the Combined DNA Index System (CODIS).

Forensic reference materials are available. The DNA Profiling Standard is composed of well-characterized human DNA in two forms: genomic DNA and DNA to be extracted from cells spotted onto filter paper.

ASSAY VALIDATION CONSIDERATIONS

The difficulty in reproducing and validating existing and emerging SNP genotyping assays due to factors such as variation in performance of PCR thermal cyclers, efficiency of different enzymes, personnel, and the presence of PCR inhibitors in the sample matrix (discussed in more detail in Nucleic Acid-Based Techniques—Amplification (1127) for general NAT assays) can hamper appropriate implementation of the technologies. Also, in the clinical laboratory the use of in-house assay formats often makes comparisons between laboratories difficult. Incorrect diagnosis of a genetic mutation can have significant consequences, so accuracy of 99.99% or higher is essential for such assays. To determine the accuracy of a technology, the new procedure should be validated on multiple samples in which the genotype has been previously determined with a gold standard procedure, such as sequencing. Even with the most accurate procedure of analysis, sample preparation and amplification and detection procedures must be optimized to eliminate any potential inaccuracies.

Some genotyping errors can be minimized by careful planning of the laboratory procedures, the inclusion of well-defined controls, and increased automation. However, errors due to the processes used for genotyping are sometimes difficult to
overcome and need to be taken into account. The types of errors and the frequency with which they occur differ between
different approaches. Situations in which preferential amplification of one allele or nonspecific probe hybridization occur can
all result in SNP micsalls. Additional unanticipated polymorphisms present within the primer/probe sequences can lead to
amplification bias, highlighting the need for careful assay design and validation using alternative techniques. Limited and
degraded samples can also result in preferential allelic amplification due to chance PCR priming events at low copy number.

It is preferable to have a no-call result, which would require the test to be repeated, than a miscall that provides incorrect
results that are subsequently reported. Performance of replicate assays may also help to ensure accuracy. Data interpretation
can also affect accuracy. Wild-type, heterozygous, and homozygous mutant results should be clearly distinguished from one
another, and a well-defined measure of uncertainty should be attributed to them. Proficiency testing schemes and ring-trials
go some way toward ensuring that individual assays are fit for the purpose for which they are intended for specific applications
and that the staff performing them are competent. Sharing of technical information for assay design and sample preparation
will also help. The availability of reference panels of well-characterized samples aids assay design and evaluation and allows
sound interlaboratory comparisons to be made.

\section{Nucleic Acid-Based Techniques—Approaches for
Detecting Trace Nucleic Acids (Residual DNA Testing)}

\section*{Introduction}

The basic principles of nucleic acid amplification technologies (NAT) and definitions of the various techniques are covered in \textit{Nucleic Acid-Based Techniques—General} (1125). This chapter covers the analytical procedures used to quantify residual DNA
in biopharmaceuticals.

Process characterization and the theoretical safety concerns associated with process-related impurities highlight the need for
residual DNA testing in biopharmaceutical products. The ability of a manufacturing process to remove residual DNA from a
biopharmaceutical product is an indicator of the quality and consistency of the process and that the process is under control.
Additionally, the cells used to produce a biopharmaceutical can be sources of a range of complex, heterogeneous, and
potentially unsafe impurities, with host cell DNA among these impurities. For continuous cell lines, the potential risk of residual
DNA arises from both of its biological activities, namely infectivity and oncogenicity. Infectivity could be due to the presence
of an infectious viral genome in the cellular DNA of the cell substrate. The oncogenicity activity of residual DNA could arise
through its capacity to induce a normal cell to become transformed, which may lead to tumorigenicity. Although animal testing
has shown that extraneous DNA can cause tumors or infections, no reports to date have demonstrated this risk in humans.

Residual DNA content, up to 10 ng of residual DNA per parenteral dose, may be considered for DNA originating from
mammalian cell cultures, but the acceptable residual DNA content may vary depending on the source of the residual DNA and
the route of administration of the product. One can address residual DNA in biopharmaceutical processes in two ways: 1) by
validating clearance during process validation; and/or 2) by monitoring residual DNA levels through routine testing of the
drug substance. Generally, 10 ng per dose is the accepted limit, by health authorities, of host residual DNA derived from mammalian
cell cultures. The level of concern regarding residual DNA can be tied to its source and the route of administration, so the
residual DNA specification and procedure for monitoring DNA clearance for a given product should be developed in consultation
with regulatory agencies. Regardless of whether routine testing of a drug substance is used to determine residual DNA content
or whether DNA clearance is demonstrated by process validation, analytical procedures for the quantification of residual DNA
are required. DNA amplification techniques, such as quantitative PCR (qPCR), are used most often for residual DNA testing
because of their superior sensitivity and unique advantages (e.g., high specificity). The expectation is that the analytical
procedure used to quantify residual DNA in biopharmaceuticals has a detection limit well below the DNA level allowed by
regulators for biopharmaceuticals (often 10 ng/dose). Assays based on hybridization, DNA-binding protein, and qPCR are
typically the techniques of choice because they can meet the sensitivity expectation.

\section*{Sample Pretreatment}

Analysis of residual DNA requires accurate quantification of picogram levels of DNA in mg (or larger) quantities of product,
which may be in a variety of matrices. In certain circumstances, the sample can be analyzed neat in the analytical procedure
with acceptable recovery and precision. When the product or other sample components interfere with the assay sample, dilution
may be all that is required to overcome the interference, so long as the specified DNA content of the sample remains within the
useful range of the analytical procedure. When sample dilution is not effective in reducing assay interference, it may be
necessary to use more extensive sample pretreatment procedures, such as proteolytic digestion, chemical dissociation, or
extractions. It may be necessary to use a combination of different pretreatment steps to remove interference to an acceptable
level. Extensive sample manipulation can lead to losses of DNA or introduction of environmental DNA, and should be a
consideration when using one or more sample pretreatment steps. Contamination with environmental DNA may only be a
concern when using a residual DNA procedure that is not sequence specific.

Protein samples may only require digestion with proteinase (e.g., Proteinase K, Pronase) to allow the analytical method to
quantitatively recover the residual DNA. It may also be possible that the DNA is bound to the sample components, and chemical
dissociation (e.g., detergents) may disrupt the binding, allowing sufficient recovery in the residual DNA assay. Residual DNA
test procedures often use protein reagents and the use of a chemical dissociation reagent. These materials must be used at a
sufficiently low level or removed so that the analysis is not compromised.

It may be necessary to extract the DNA from the sample to remove the inhibitory substances that are causing the reduced
DNA recovery. Extraction procedures are typically based on precipitating the DNA from the sample or DNA-specific binding

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to a matrix (e.g., magnetic beads). Historically, extraction methods based on phenol and chloroform, followed by ethanol precipitation, have been applied to the purification of DNA in molecular biology research. The phenol/chloroform extraction technique may be a useful pretreatment for residual DNA samples before analysis, but the phenol/chloroform extraction technique might not be the best choice for the low levels of DNA typically found in biopharmaceutical samples. Because of these low levels, quantitative DNA recovery with ethanol precipitation may be difficult. For this reason, a carrier molecule (e.g., glycogen) may be necessary to aid in DNA recovery if the phenol/chloroform extraction technique is used. Commercial kits are available and have been successfully used for pretreatment of residual DNA samples for improved recovery in the residual DNA assay. For example, some kits use a chaotrope (sodium iodide) and a detergent (sodium N-lauroyl sarcosinate) to disrupt the association of the DNA with the sample. The DNA is then co-precipitated using glycogen as the carrier molecule in the presence of isopropanol. Extraction of DNA from the sample, based on binding to a solid matrix, can be found in various formats. One of the most popular formats uses magnetic beads, where the beads are added to a sample with a binding solution to capture the sample DNA on the beads. The beads are then captured and held in the sample tube using a magnetic stand while the supernatant containing the interfering substances is removed and discarded. The beads are washed repeatedly using a magnetic stand and a wash solution. Finally, the DNA is eluted from the beads for the assay using an elution buffer, with the beads being removed from the sample preparation using the magnetic stand.

The sample manipulation involved with pretreatment may reduce the recovery of the residual DNA or introduce environmental DNA into the sample. Great care must be taken during any sample manipulations to avoid DNA losses or contamination. The addition of target DNA-spiked samples in the residual DNA assay is a common practice. The target DNA-spiked sample should not be confused with the internal positive control (IPC), which is typically a nontarget DNA added after the sample pretreatment step to detect the presence of PCR inhibitors and to evaluate DNA amplification during the analysis. The IPC may also be introduced before the extraction to improve the control of this step. A recovery of 50%–150% of the spiked target DNA is often applied to residual DNA assays to ensure that the assay yields acceptable results. When sample characteristics (e.g., matrix effects or sample preparation method) make achieving a recovery acceptance criterion of 50%–150% impractical, then correcting the observed DNA concentration using the load recovery percentage is also an acceptable approach.

**HYBRIDIZATION-BASED RESIDUAL DNA ASSAY**

The first residual DNA assays were based on DNA hybridization, wherein a DNA probe created from host cell DNA detects and quantifies the amount of complementary DNA present in the product under assay. Double-stranded host cell DNA consists of two complementary strands of DNA that are held together by hydrogen bonding. The double-stranded DNA in the test sample is denatured to single strands and immobilized to a membrane, typically a nitrocellulose or nylon membrane. The sample is probed using host cell DNA that has been denatured and labeled. The host cell DNA probe is not a specific sequence but is prepared by a random labeling procedure during which a radioactive or fluorescent label is introduced into the host cell DNA to produce the probe. When the denatured, labeled DNA probe is brought into contact with the membrane-immobilized DNA, the probe will bind to complementary sequences of the host cell DNA. If the probe is radioactive, the membrane is placed against a film of photographic emulsion and exposed for a sufficient length of time, the film is developed, and a dark spot will be observed where the test DNA was immobilized. The level of hybridization can be measured using a phosphor-imaging system. If the probe has a fluorescent label, the intensity of the spots is determined using a fluorescence-imaging system. The intensity of the spot is proportional to the amount of probe that was hybridized to the test DNA and therefore is proportional to the amount of residual DNA in the sample. The intensity of the spot can be compared visually with the intensity of spots that correspond to a standard curve yielding semiquantitative results (i.e., visual quantitation), or the intensity can be determined using an instrument (e.g., densitometer) to create a quantitative value that is compared with the values obtained from the standard curve.

**DNA-BINDING PROTEIN-BASED RESIDUAL DNA ASSAY**

Instrumentation is commercially available for the quantitation of residual DNA in biopharmaceuticals. The instrumentation requires reagents that use DNA-binding protein and antibodies targeted for DNA in a four-step analytical procedure.

1. The first step requires that the DNA be denatured into single-stranded DNA by sample heating. The denatured DNA is mixed with a single reagent that contains DNA-binding protein that is conjugated with streptavidin and a monoclonal anti-DNA antibody that is conjugated to urease. The DNA-binding protein and the monoclonal antibody are specific for single-stranded DNA but do not have any sequence specificity. This liquid phase facilitates the formation of reaction complexes that contain DNA, streptavidin, and urease.

2. During the second step, the sample is filtered through a biotinylated membrane that binds to the streptavidin and captures the complexes on the membrane, which is washed to remove any reagents that are not bound to the membrane.

3. During the third step, the membrane is inserted into a sensor on the instrument, where the urease in the DNA complex reacts with a urea solution in the sensor, producing ammonia and a change in pH that is detected using a light-addressable potentiometric sensor (LAPS). The change in pH directly correlates with the amount of DNA in the sample.

4. In the fourth step, the raw data from the instrument are analyzed using the appropriate software to determine the residual DNA content of the sample.

**POLYMERASE CHAIN REACTION TECHNIQUES**

Real-time qPCR is a procedure that is well adapted to fast sample throughput and has applications in many areas of biopharmaceutical manufacture (e.g., copy number detection, virus detection). The technique can quantify the amount of a nucleic acid target sequence in DNA from a variety of samples. The DNA probe and primers used in the analysis are key to the analysis.
procedure. The most common qPCR method for detection of this amplification is referred to as the 5’ nuclease assay. In this format, the probe has a reporter dye attached to one end and a quencher dye attached to the other end. A pair of DNA primers is also added to the reaction. During the amplification reaction, a thermostable DNA polymerase initiates DNA synthesis where the DNA primer binds to the single-stranded sample (template) DNA and moves along the sample DNA, synthesizing new complementary DNA. While following the template DNA, DNA polymerase cleaves any complementary DNA in the path. If DNA polymerase I encounters the labeled DNA probe, DNA polymerase I will cleave the probe. The reporter dye is released into the solution and, in the absence of the quencher dye, the resulting fluorescence is measured. Repeating the reaction cycle results in an amplification of the fluorescence signal. The number of cycles required for the fluorescence measurement to exceed a threshold value correlates to the amount of starting residual DNA in the sample. By comparing the fluorescence obtained from a sample to a standard curve, analysts can quantify the residual DNA in the sample.

Alternative Detection Strategies

A number of innovative detection strategies have been developed and commercialized beyond that described above. A few of the most common are as follows:

1. Intercalating cyanine dyes fluoresce after binding to double-stranded DNA. The amount of dye incorporated is proportional to the amount of target amplicon generated. These dyes are inexpensive and easy to use. The disadvantage of this technique is the lack of a specific probe to confer sequence specificity beyond that afforded by the primers, and the dye will also bind somewhat to single-stranded DNA and RNA molecules. Consequently, primer dimers or nonspecific products may affect the quantification. However, it is possible to check for the specificity of the system by running a melting curve at the end of the PCR run, based on the principle that every product has a different dissociation temperature and depending on the size and base content.

2. Other sensitive probes exist that contain a stem-loop structure with a fluorophore and a quencher at their 5’ and 3’ ends, respectively. The stem is usually six bases long, mainly consisting of cytosines and guanines, and holds the probe in the hairpin configuration. The “stem” sequence keeps the fluorophore and the quencher in close proximity, but only in the absence of a sequence complementary to the “loop” sequence. In the presence of a complementary sequence, the probe unfolds and hybridizes to the target, leading to separation between the fluorophore and the quencher, and the probe fluoresces. The amount of signal is proportional to the amount of target amplicon sequence. The increase in fluorescence that occurs is reversible, because there is no cleavage of the probe. It is also possible to design the stem structure to add specificity to this type of probe. However, these probes are often expensive, and the signal can be weak due to the limited possible physical separation between the fluorophore and the quencher.

3. A variation on the second example described above uses a single-stranded nucleic acid sequence containing the specific PCR primers, the specific probe with a stem-loop tail separating a fluorophore and a quencher, and a blocking group. The stem-loop tail is separated from the PCR primer sequence by a “PCR blocker”, a chemical modification that prevents the polymerase from copying the stem-loop sequence of the primer. After extension of the primer during PCR amplification, the specific probe sequence is able to bind to its complement within the same strand of DNA. This hybridization event opens the hairpin loop so that fluorescence is no longer quenched, and an increase in signal is observed. Covalent attachment of the probe to the target amplicon ensures that each probe has a target in close proximity. Enzymatic cleavage is not required, nor a separate probe hybridization step, thereby reducing the time needed for signaling. This can result in stronger signals and lower background with faster cycling; however, these probes can be quite expensive and complicated to design.

4. Fluorogenic minor groove binder probes are short linear probes that have a minor groove binder with a nonfluorescent quencher on the 5’ end and a fluorophore on the 3’ end. The minor groove binder prevents the exonuclease activity of the DNA polymerase from cleaving the probe. Quenching occurs when the random coiling of the probe in the free form brings the quencher and the fluorophore close together. The probe is elongated when bound to its target and quenching is decreased, leading to an increase in fluorescent signal proportional to the amount of accumulated amplicon. These probes are also expensive and can produce a low signal-to-noise ratio.

Quantitative Multiplex PCR-Based Residual DNA Assay

An evolution of qPCR is quantitative multiplex PCR, where several pairs of primers and the corresponding probes are introduced in the reaction medium to simultaneously detect multiple targets. Benefits include higher throughput and better control of false-negative results, whereas disadvantages come from amplification and detection interferences, as outlined in Nucleic Acid-Based Techniques—Amplification (1127). One of the applications of this technique is a duplex qPCR, where the introduction of an exogenous DNA, called IPC (internal positive control, see Sample Pretreatment above), enhances confidence in the accuracy of the analysis when appropriately amplified. Multiplex qPCR is not used as often as single-target qPCR to assay host cell residual DNA in biopharmaceuticals.

RESIDUAL DNA TESTING POINTS TO CONSIDER

When developing a residual DNA assay, one should consider how the assay will be used, the structure of the DNA available (e.g., fragment length), and regulatory issues. The cost of analysis can be significant and should be considered when evaluating an assay format. In addition, environmental, health, and safety aspects should be considered. Traditionally, hybridization assays were performed using phosphorus (32P)-labeled DNA and autoradiography. Because 32P decays quickly, probes prepared with 32P have a limited shelf life, and the precautions necessary for handling radioactive material can be cumbersome. These issues with 32P labeling may make fluorescence labeling of the hybridization probe a more desirable option. If the hybridization assay is assessed visually, this process represents a semiquantitative assay, but if the intensity of the spots is determined using a
dosing regimen. These considerations, organized by route of administration, are detailed in general chapters and testing of all dosage forms target drug product quality. A testing protocol must consider not only the physical, chemical, microbiological, and biological properties of the dosage form as appropriate, but also the administration route and desired scheduling consideration when selecting an assay. Procedures exist to determine whether the DNA fragments in a sample are too small for adequate residual DNA recovery with a given assay. When bridging from one DNA assay technique to another, a thorough understanding of the DNA analyte is critical. Some assays can detect both single-stranded and double-stranded DNA, whereas some can only detect double-stranded DNA (e.g., some fluorescent dye-binding assays). There are assays that are not sequence specific, and those assays that are sequence specific can be influenced by the copy number of the target sequence present in the DNA. There are assays that require two or more antibody molecules to bind to the DNA fragment (e.g., DNA-binding protein-based residual DNA assay), and if the DNA fragments are too small and present in sufficient quantity, they can saturate the reagents and inhibit the assay (hook effect).

Although safety concerns regarding residual DNA impurities are not as prominent as they once were, the levels of residual DNA in any bioprocess remain a key quality attribute and provides valuable characterization of the manufacturing process.

### 1151 PHARMACEUTICAL DOSAGE FORMS

#### GENERAL CONSIDERATIONS

This chapter provides general descriptions of and definitions for drug products, or dosage forms, commonly used to administer the drug substance (active pharmaceutical ingredient; API). It discusses general principles involved in the manufacture or compounding of these dosage forms. A glossary is provided as a nomenclature resource and should be used in conjunction with the Nomenclature Guidelines.¹

A dosage form is a pharmaceutical preparation consisting of drug substance(s) and/or excipient(s) to facilitate dosing, administration, and delivery of the content of the drug product or placebo to the patient. The design, materials, manufacturing, and testing of all dosage forms target drug product quality. A testing protocol must consider not only the physical, chemical, microbiological, and biological properties of the dosage form as appropriate, but also the administration route and desired dosing regimen. These considerations, organized by route of administration, are detailed in general chapters Injections and Implanted Drug Products (Parenterals)—Product Quality Tests (1), Oral Drug Products—Product Quality Tests (2), Topical and Transdermal Drug Products—Product Quality Tests (3), Mucosal Drug Products—Product Quality Tests (4), Inhalation and Nasal Drug Products—General Information and Product Quality Tests (5), and Ophthalmic Products—Quality Tests (771).² The organization of this general information chapter is by the quality attributes of each particular dosage form, generally without specific reference to the route of administration. The list below provides the preferred dosage forms used in official article titles. In addition to the preferred dosage forms, the Glossary contains other terms that have been used in current official article titles but are not preferred and should not be used for new drug product titles.

**Official Dosage Forms Used in Official Article Titles**

- Aerosols
- Capsules
- Creams
- Emulsions
- Films
- Foams
- Gases
- Gels
- Granules
- Gums
- Implants
- Injections
- Inserts
- Irritations
- Liquids
- Lotions
- Lozenges
- Ointments
- Pastes
- Pellets
- Pills
- Powders
- Rinses
- Shampoos
- Soaps
- Solutions
- Sprays
- Strips
- Suppositories
- Suspensions
- Systems
- Tablets

Tests to ensure compliance with USP standards for dosage form performance fall into one of the following areas.


Dose Uniformity

Consistency in dosing for a patient or consumer requires that the variation in the drug substance content of each dosage unit be accurately controlled throughout the manufactured batch or compounded lot of drug product. Uniformity of dosage units typically is demonstrated by one of two procedures: content uniformity or weight variation. The procedure for content uniformity requires the appropriate assay of the drug substance content of individual units. The procedure for weight variation uses the weight of the individual units to estimate their content. Weight variation may be used where the underlying distribution of the drug substance in the blend is presumed to be uniform and well-controlled, as in solutions. In such cases, the content of the drug substance may be adequately estimated by the net weight. Content uniformity does not rely on the assumption of blend uniformity and can be applied in all cases. Successful development and manufacture of dosage forms requires careful evaluation of the drug substance particle or droplet size, incorporation techniques, and excipient properties.

Stability

Drug product stability involves the evaluation of chemical stability, physical stability, and performance over time. The chemical stability of the drug substance in the dosage form must support the expiration dating for the commercially prepared dosage forms and a beyond-use date for a compounded dosage form. Test procedures for potency must be stability indicating (see Validation of Compendial Procedures (1225)). Degradation products should be quantified. In the case of dispersed or emulsified systems, consideration must be given to the potential for settling or separation of the formulation components. Any physical changes to the dosage form must be easily reversed (e.g., by shaking) prior to dosing or administration. For tablets, capsules, oral suspensions, and implants, in vitro release test procedures such as dissolution and disintegration provide a measure of continuing consistency in performance over time (see Dissolution (711), Disintegration (701), and Drug Release (724)).

Bioavailability

Bioavailability is influenced by factors such as the method of manufacture or compounding, particle size, crystal form (polymorph) of the drug substance, the properties of the excipients used to formulate the dosage form, and physical changes as the drug product ages. Assurance of consistency in bioavailability over time (bioequivalence) requires close attention to all aspects of the production (or compounding) and testing of the dosage form. With proper justification, in vitro release testing (e.g., disintegration and dissolution) may be used as a surrogate to demonstrate consistent availability of the drug substance from the formulated dosage.

Release Profile

Two principal categories of drug release are recognized: immediate-release and modified-release. “Immediate-release” is observed when no deliberate effort has been made to modify the drug substance release profile. For example, capsules and tablets are considered immediate-release even if a disintegrating agent or a lubricant has been used. “Modified-release” is a term used when the rate and/or time of release of the drug substance is altered as compared to what would be observed or anticipated for an immediate-release product. Two modified-release profiles, delayed-release and extended-release, are recognized. The term “modified-release” is not used for official article titles. “Delayed-release” is used when deliberate formulation achieves a delay in the release of the drug substance for some period of time after initial administration. For oral products, expressions such as “enteric-coated” or “gastro-resistant” have also been used where release of the drug substance is prevented in the gastric environment but promoted in the intestinal environment. However, the term “delayed-release” is used for official article titles. “Extended-release” is used when the deliberate formulation achieves prolongation of drug substance release compared to that observed or anticipated for an immediate-release dosage form. Expressions such as “prolonged-release”, “repeat-action”, “controlled-release”, “long-acting”, and “sustained-release” have also been used to describe such dosage forms. However, the term “extended-release” is used for official article titles.

The Nomenclature Guidelines\(^3\) should be consulted for naming conventions for products with a single drug substance or for products with a combination of more than one drug substance displaying the combination of release profiles of immediate-release and extended-release, immediate-release and delayed-release, or extended-release and delayed-release.

Manufacture

Although detailed instructions about the manufacture of any of these dosage forms are beyond the scope of this general information chapter, general manufacturing principles have been included.\(^3\) Information relative to extemporaneous compounding of dosage forms can be found in Pharmaceutical Compounding—Nonsterile Preparations (795) and Pharmaceutical Compounding—Sterile Preparations (797).

Route of Administration

The primary routes of administration for pharmaceutical dosage forms can be defined as parenteral (see (1)), gastrointestinal (see (2)), topical (see (3)), mucosal, and (see (4)), inhalation (see (5)). Each has subcategories as needed. Many tests used to

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\(^3\) The terms “manufacture” and “preparation” are used interchangeably in this general chapter.
ensure quality generally are applied across all of the administration routes, but some tests are specific for individual routes. For example, products intended for injection must be evaluated using Sterility Tests (71), Bacterial Endotoxins Test (85), or Pyrogen Test (151), and the manufacturing process (and sterilization technique) employed for parenterals (by injection) should ensure compliance with these tests. Tests for particulate matter may be required for certain dosage forms depending on the route of administration (e.g., by injection—Particulate Matter in Injections (788), or mucosal—Particulate Matter in Ophthalmic Solutions (789)). Additionally, dosage forms intended for the inhalation route of administration must be monitored for particle size and spray pattern (for a metered-dose inhaler or dry powder inhaler) and droplet size (for nasal sprays). Further information regarding administration routes and suggested testing can be found in the Guide to General Chapters, Chapter Charts, Charts 4–8, 10, and 13.

An appropriate manufacturing process and testing regimen helps ensure that a dosage form can meet the appropriate quality attributes for the intended route of administration.

Packaging and Storage

Suitable packaging is determined for each product. For additional information about meeting packaging requirements listed in the individual labeling, refer to Packaging and Storage Requirements (659), Containers—Performance Testing (671), and Good Repackaging Practices (1178). Product labeling must specify storage requirements that describe environmental conditions, limitations, and restrictions. For instance, exposure to excessive temperature, humidity, and light can influence the ability of the packaging to protect the product.

Labeling Statements

Some dosage forms or articles have mandatory labeling statements that are given in the Code of Federal Regulations (e.g., 21 CFR §201.320 and 21 CFR §369.21). The text of 21 CFR should be consulted to determine the current recommendations.

Change to read:

PRODUCT QUALITY TESTS, GENERAL

International Council for Harmonisation (ICH) Guidance Q6A (available at www.ich.org) recommends specifications (list of tests, references to analytical procedures, and acceptance criteria) to ensure that drug products are safe and effective at the time of release and over their shelf life. Tests that are universally applied to ensure safety, efficacy, strength, quality, and purity include description, identification, assay, and impurities.

Description

The Definition section (see General Notices, 4.10 Monographs) in a USP monograph describes the drug product and specifies the range of acceptable assayed content of the drug substance(s) present in the dosage form. For certain products, the Definition includes any relevant additional information, such as the presence or absence of other components, excipients, or adjuvants, cautionary statements on toxicity and stability, etc. While appearance information to aid in identification is used in a regulatory submission (e.g., a qualitative description of size, shape, color, etc.) it is typically not required as part of a USP monograph. This information is drug product specific.

Identification

Identification tests are discussed in General Notices, 5.40 Identification. Identification tests should establish the identity of the drug substance(s) present in the drug product and should discriminate between compounds of closely related structure that are likely to be present. Identification tests should be specific for the drug substance(s). For example, the infrared absorption spectrum is often used (see Mid-Infrared Spectroscopy (854) and Spectroscopic Identification Tests (197)). If no suitable infrared spectrum can be obtained, other analytical methods can be used. Near-infrared (NIR) or Raman spectrophotometric methods could also be acceptable as the sole identification method of the drug product formulation (see Near-Infrared Spectroscopy—Theory and Practice (1856) and Raman Spectroscopy—Theory and Practice (1858). Identification by a chromatographic retention time from a single procedure is not regarded as specific. The use of retention times from two chromatographic procedures for which the separation is based on different principles or a combination of tests in a single procedure can be acceptable (see Chromatography (621) and Thin-Layer Chromatographic Identification Test (201)).

Assay

A specific and stability-indicating test should be used to determine the strength (drug substance content) of the drug product. Some examples of these procedures are Antibiotics—Microbial Assays (81), (621), or Assay for Steroids (351). In cases when the use of a nonspecific assay is justified (e.g., Titrimetry (541)), other supporting analytical procedures should be used to achieve specificity. When evidence of excipient interference with a nonspecific assay exists, a procedure with demonstrated specificity should be used.
Impurities

Process impurities, synthetic byproducts, and other inorganic and organic impurities may be present in the drug substance and excipients used in the manufacture of the drug product. These impurities are evaluated by tests in the drug substance and excipients monographs. Impurities arising from degradation of the drug substance or from the drug-product manufacturing process should be monitored. Residual Solvents (467) is applied to all products where relevant. In some cases, testing for heavy metal impurities is appropriate. In addition to the universal tests listed, the following tests may be considered on a case-by-case basis.

Physicochemical Properties

Examples include pH (791), Viscosity—Capillary Methods (911) or Viscosity—Rotational Methods (912), and Specific Gravity (841).

Particle Size

For some dosage forms, particle size can have a significant effect on dissolution rates, bioavailability, therapeutic outcome, and stability. Procedures such as those found in Inhalation and Nasal Drug Products: Aerosols, Sprays, and Powders—Performance Quality Tests (601) and Particle Size Distribution Estimation by Analytical Sieving (786) could be used.

Uniformity of Dosage Units

See the discussion of Dose Uniformity in the General Considerations section.

Water Content

A test for water content is included when appropriate (see Water Determination (921)).

Microbial Limits

The type of microbial test(s) and acceptance criteria are based on the nature of the nonsterile drug product, method of manufacture, and the route of administration (see Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests (61), Microbiological Examination of Nonsterile Products: Tests for Specified Microorganisms (62), and Microbiological Examination of Nonsterile Products: Acceptance Criteria for Pharmaceutical Preparations and Substances for Pharmaceutical Use (1111)).

Antimicrobial Preservative Content

Acceptance criteria for preservative content in multidose products should be established. They are based on the levels of antimicrobial preservative necessary to maintain the product’s microbiological quality at all stages throughout its proposed usage and shelf life (see Antimicrobial Effectiveness Testing (51)).

Antioxidant Content

If antioxidants are present in the drug product, tests of their content should be performed to maintain the product’s quality at all stages throughout its proposed usage and shelf life.

Sterility

Depending on the route of administration (e.g., ophthalmic preparations, implants, aqueous-based preparations for oral inhalation, and injections) sterility of the product is demonstrated as appropriate (see 71).

Dissolution

A test to measure the release of the drug substance(s) from the drug product normally is included for dosage forms such as tablets, capsules, suspensions, granules for suspensions, implants, transdermal delivery systems, and medicated chewing gums. Single-point measurements typically are used for immediate-release dosage forms. For modified-release dosage forms, appropriate test conditions and sampling procedures are established as needed (see 711 and 724). In some cases, dissolution testing may be replaced by disintegration testing (see 701).

Breaking Force and Friability

These parameters are evaluated as in-process controls. Acceptance criteria depend on packaging, supply chain, and intended use (see Tablet Friability (1216) and Tablet Breaking Force (1217)).
Leachables

When evidence exists that leachables from the container–closure systems (e.g., rubber stopper, cap liner, or plastic bottle) have an impact on the safety or efficacy of the drug product, a test is included to evaluate the presence of leachables.

Other Tests

Depending on the type and composition of the dosage form, other tests such as alcohol content, redispersibility, particle size distribution, rheological properties, reconstitution time, endotoxins/pyrogens, particulate matter, functionality testing of delivery systems, delivered dose uniformity, viscosity, and osmolarity may be necessary.

DOSAGE FORMS

Aerosols

Aerosols are dosage forms packaged under pressure and contain therapeutic agent(s) and a propellant that are released upon actuation of an appropriate valve system. Upon actuation of the valve system, the drug substance is released as a plume of fine particles or droplets. Only 1 dose is released from the preparation upon actuation of a metered valve. In the case of topical products and depending on the nature of the drug substance and the conditions being treated, actuation of the valve may result in a metered release of a controlled amount of the formulation or the continuous release of the formulation as long as the valve is depressed.

The aerosol dosage form refers only to those products packaged under pressure that release a fine mist of particles or droplets when actuated (see Glossary). Other products that produce dispersions of fine droplets or particles will be covered in subsequent sections (e.g., Powders and Sprays).

TYPICAL COMPONENTS

Typical components of aerosols are the formulation containing one or more drug substance(s) and propellant, the container, the valve, and the actuator. Each component plays a role in determining various characteristics of the emitted plume, such as droplet or particle size distribution, uniformity of delivery of the therapeutic agent, delivery rate, and plume velocity and geometry. The metering valve and actuator act in tandem to generate the plume of droplets or particles. The metering valve delivers an accurate volume of the pressurized liquid formulation from the container. The actuator directs the metered volume to a small orifice that is open to the atmosphere. Upon actuation, the formulation is forced through the opening, forming the fine mist of particles that are directed to the site of administration.

Aerosol preparations may consist of either a two-phase (gas and liquid) or a three-phase (gas, liquid, and solid or liquid) formulation. The two-phase formulation consists of drug substance(s) dissolved in liquefied propellant. Co-solvents such as alcohol may be added to enhance the solubility of the drug substance(s). Three-phase inhalation and nasal aerosol systems consist of suspended drug substance(s) in propellant(s), co-solvents, and potentially other suitable excipients. The suspension or emulsion of the finely divided drug substance is typically dispersed in the liquid propellant with the aid of suitable biocompatible surfactants or other excipients.

Propellants for aerosol formulations are typically low molecular weight hydrofluorocarbons or hydrocarbons that are liquid when constrained in the container, exhibit a suitable vapor pressure at room temperature, and are biocompatible and nonirritating. Compressed gases do not supply a constant pressure over use and typically are not used as propellants. Metal containers can withstand the vapor pressure produced by the propellant. Excess formulation may be added to the container to ensure that the full number of labeled doses can be accurately administered. The container and closure must be able to withstand the pressures anticipated under normal use conditions as well as when the system is exposed to elevated temperatures.

TYPES OF AEROSOL DOSAGE FORMS

Aerosol dosage forms can be delivered via various routes. The container, actuator, and metering valve, as well as the formulation, are designed to target the site of administration.

Inhalation aerosols, commonly known as metered-dose inhalers (MDIs), are intended to produce fine particles or droplets for inhalation through the mouth and deposition in the pulmonary tree. The design of the delivery system is intended to release measured mass and appropriate quality of the active substance with each actuation.

Nasal aerosols, commonly known as nasal MDIs, produce fine particles or droplets for delivery through the nasal vestibule and deposition in the nasal cavity. Each actuation of the valve releases a measured mass of the drug substance with appropriate quality characteristics.

Lingual aerosols are intended to produce fine particles or droplets for deposition on the surface of the tongue. The design of the delivery system releases 1 dose with each actuation.

Topical aerosols produce fine particles or droplets for application to the skin.

LABELING FOR PROPER USE

Refer to 21 CFR §201.320 and 21 CFR §369.21.
Capsules

Capsules are solid dosage forms in which the drug substance and/or excipients are enclosed within a soluble container or shell or coated on the capsule shell. The shells may be composed of two pieces (a body and a cap), or they may be composed of a single piece. Two-piece capsules are commonly referred to as hard-shell capsules, and one-piece capsules are often referred to as soft-shell capsules. This two-piece and one-piece capsule distinction, although imprecise, reflects differing levels of plasticizers in the two compositions and the fact that one-piece capsules typically are more pliable than two-piece capsules.

The shells of capsules are usually made from gelatin. However, they may also be made from cellulose polymers or other suitable material. Most capsules are designed for oral administration. When no deliberate effort has been made to modify the drug substance release rate, capsules are referred to as immediate-release.

TWO-PIECE OR HARD-SHELL CAPSULES

Two-piece capsules consist of two telescoping cap and body pieces in a range of standard sizes.

ONE-PIECE OR SOFT-SHELL CAPSULES

One-piece capsules typically are used to deliver a drug substance as a solution or suspension. Liquid formulations placed into one-piece capsules may offer advantages by comparison with dry-filled capsules and tablets in achieving content uniformity of potent drug substance(s) or acceptable dissolution of drug substance(s) with poor aqueous solubility. Because the contact between the shell wall and its liquid contents is more intimate than in dry-filled capsules, undesired interactions may be more likely to occur (including gelatin crosslinking and pellicle formation).

MODIFIED-RELEASE CAPSULES

The release of drug substance(s) from capsules can be modified in several ways. There are two categories of modified-release capsule formulations recognized by USP.

Delayed-release capsules: Capsules are sometimes formulated to include enteric-coated granules to protect acid-labile drug substances from the gastric environment or to prevent adverse events such as irritation. Enteric-coated multiparticulate capsule dosage forms may reduce variability in bioavailability associated with gastric emptying times for larger particles (i.e., tablets) and to minimize the likelihood of a therapeutic failure when coating defects occur during manufacturing. Alternatively, a coating may be applied to the capsule shell to achieve delayed release of the contents.

Extended-release capsules: Extended-release capsules are formulated in such a manner as to make the contained drug substance available over an extended period of time following ingestion. Requirements for dissolution (see (711)) are typically specified in the individual monograph.

Methods for modifying drug substance release from capsules include coating the filled capsule shells or the contents, in the case of dry-filled capsules.

PREPARATION

Two-piece capsules: Two-piece gelatin capsules are usually formed from blends of gelatins that have relatively high gel strength in order to optimize shell clarity and toughness or from hypromellose. They may also contain colorants such as Drug & Cosmetic (D&C) and Food, Drug, & Cosmetic (FD&C) dyes or various pigments, opaquing agents such as titanium dioxide, dispersing agents, plasticizers, and preservatives. Gelatin capsule shells normally contain between 12% and 16% water.

The shells are manufactured in one set of operations and later filled in a separate manufacturing process. Two-piece shell capsules are made by a process that involves dipping shaped pins into gelatin or hypromellose solutions, followed by drying, cutting, and joining steps.

Powder formulations for two-piece gelatin capsules generally consist of the drug substance and at least one excipient. Both the formulation and the method of filling can affect release of the drug substance. In the filling operation, the body and cap of the shell are separated before filling. Following the filling operation, the machinery rejoins the body and cap and ensures satisfactory closure of the capsule by exerting appropriate force on the two pieces. The joined capsules can be sealed after filling by a band at the joint of the body and cap or by a designed locking joint between the cap and body. In compounding prescription practice, two-piece capsules may be hand-filled. This permits the prescriber the choice of selecting either a single drug substance or a combination of drug substances at the exact dose level considered best for an individual patient.

One-piece capsules: One-piece capsules are formed, filled, and sealed in a single process on the same machine and are available in a wide variety of sizes, shapes, and colors. The most common type of one-piece capsule is that produced by a rotary die process that results in a capsule with a seam. The soft gelatin shell is somewhat thicker than that of two-piece capsules and is plasticized by the addition of polyols such as glycerin, sorbitol, or other suitable materials. The ratio of the plasticizer to the gelatin can be varied to change the flexibility of the shell depending on the nature of the fill material, its intended usage, or environmental conditions.

4 In 1960 Congress enacted the Color Additive Amendments, requiring the FDA to regulate dyes, pigments, or other coloring agents in foods, drugs, and cosmetics separately from food additives. Under the law, color additives are deemed unsafe unless they are used in compliance with FDA regulations. The law provides a framework for the listing and certification of color additives. See FDCA §721; see FDA regulations at 21 CFR Part 70. Colors must also be listed in pertinent FDA regulations for specific uses; the list of color additives for drugs that are exempt from certification is published at 21 CFR Part 73, Subpart B. FDA also conducts a certification program for batches of color additives that are required to be certified before sale; see 21 CFR Part 74 (Subpart B re: drugs). Regulations regarding certification procedures, general specifications, and the listing of certified provisionally listed colors are at 21 CFR Part 80. FDA maintains a color additives website with links to various legal and regulatory resources at: http://www.fda.gov; search by document title.

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In most cases, one-piece capsules are filled with liquids. Typically, drug substances are dissolved or suspended in a liquid vehicle. Classically, an oleaginous vehicle such as a vegetable oil was used. However, nonaqueous, water-miscible liquid vehicles such as the lower molecular weight polyethylene glycols are now more common. The physicochemical properties of the vehicle can be chosen to ensure stability of the drug substance as well as to influence the release profile from the capsule shell.

Creams

(See Emulsions.)

Emulsions

An emulsion is a dispersed colloidal system consisting of two immiscible liquid phases generally stabilized with one or more suitable agents.

Typical pharmaceutical emulsions are prepared from immiscible aqueous and organic (oil) liquids. Complex multiple-phase systems may exist in an emulsion. Whether the organic or the aqueous phase is the dispersed phase depends on the volumes of the two phases, the emulsifier chosen, and the method of preparation. When an oil phase is dispersed in an aqueous phase, the emulsion is termed an oil-in-water (O/W) emulsion and water is referred to as the continuous phase. When water is dispersed in oil, the emulsion is referred to as a water-in-oil (W/O) emulsion. Emulsions have dispersed phases typically ranging from 0.1 to 100 µm. Emulsions are opaque while microemulsions are usually transparent or translucent. Microemulsions have dispersed phases less than 0.1 µm.

Emulsions may exhibit three types of instability: flocculation, creaming, and coalescence. Flocculation describes the process by which the dispersed phase comes out of suspension in the form of flakes. Coalescence is another form of instability—small droplets within the media continuously combine to form progressively larger droplets. Emulsions can also undergo creaming, where one of the phases migrates to the top (or the bottom, depending on the relative densities of the two phases) of the emulsion. To prevent flocculation, creaming, and coalescence of the emulsions, manufacturers commonly add surfactants, pH-modifying agents, or emulsifying agents to increase the stability of emulsions so that the emulsion does not change significantly with time.

Emulsions are widely used as pharmaceutical dosage forms. Oral emulsions have been prepared to improve taste, solubility, stability, or bioavailability. Emulsions for topical administration are referred to as creams, lotions, and sometimes ointments. Parenteral emulsions have been used for anesthetics, parenteral nutrition, and to deliver poorly water-soluble drugs.

CREAMS

Creams are semisolid emulsion dosage forms. They often contain more than 20% water and volatiles, and/or typically contain less than 50% hydrocarbons, waxes, or polyols as the vehicle for the drug substance. Creams are generally intended for external application to the skin or to the mucous membranes. Creams have a relatively soft, spreadable consistency and can be formulated as either a W/O emulsion (e.g., Cold Cream or Fatty Cream as in the European Pharmacopoeia) or as an oil-in-water emulsion (e.g., Betamethasone Valerate Cream). Creams are generally described as either nonwashable or washable, reflecting the fact that an emulsion with an aqueous external continuous phase is more easily removed than one with a nonaqueous external phase (W/O emulsion).

LOTIONS

Lotions are an emulsified liquid dosage form intended for external application to the skin. Historically, some topical suspensions such as calamine lotion have been called lotions but that nomenclature is not currently preferred. Lotions share many characteristics with creams. The distinguishing factor is that they are more fluid than semisolid and thus pourable. Due to their fluid character, lotions are more easily applied to large skin surfaces than semisolid preparations. Lotions may contain antimicrobial agents as preservatives.

INJECTABLE EMULSIONS

Injectable emulsions are sterile liquid dosage forms of drug substances dissolved or dispersed in a suitable emulsion medium. Injectable emulsions are for parenteral administration of poorly water-soluble drugs.

OINTMENTS

Ointments are sometimes semisolid emulsion dosage forms (see Dosage Forms, Ointments).

PREPARATION

Chapter (795) provides general information regarding the preparation of emulsions. Creams: Creams may be formulated from a variety of oils, both mineral and vegetable, and from fatty alcohols, fatty acids, and fatty esters. Emulsifying agents include nonionic surfactants, detergents, and soaps. Soaps are usually formed in situ during the preparation of creams from a fatty acid in the oil phase hydrolyzed by a base dissolved in the aqueous phase.

Preparation usually involves separating the formula components into two portions: lipid and aqueous. The lipid portion contains all water-insoluble components and the aqueous portion contains the water-soluble components. Both phases are heated to a temperature above the melting point of the highest melting component. The phases are then mixed and the mixture is stirred until reaching ambient temperature or until the mixture has congealed. Mixing is generally continued during the cooling process to promote uniformity. Traditionally, the aqueous phase is added to the lipid phase, but comparable results...
and suitable excipients. Typical excipients intended for foam dosage forms include surfactants to ensure distribution of the gas/propellant in the formulation, aqueous or nonaqueous vehicles, and propellants (for pressurized systems). Foams are produced by mechanical means or via interaction of propellant gas and the formulation under pressure. For example, emulsions intended for intravenous administration should comply with *Globule Size Distribution in Lipid Injectable Emulsions* (729). The procedure to assure sterility should be validated by media fills. Preservatives are generally not used in injectable emulsions.

**Ointments:** (See *Dosage Forms, Ointments.*)

**Films**

Films are thin sheets that are placed in the oral cavity. They contain one or more layers. A layer may or may not contain the drug substance. Typically, these thin sheets are formed by casting or extrusion that results in a dispersion of the components through the film. Films are classified by the site of application. “Oral films” can be formulated to deliver medication to the mouth such as oral hygiene products or to deliver medication to the gastrointestinal tract for absorption. “Buccal films” and “sublingual films” are formulated to facilitate absorption through the proximal mucosal membranes avoiding first pass metabolism or degradation in the gastrointestinal tract and providing a quick onset of action. Films can be formulated with edible polymers such as pullulan or with water-soluble polymers such as modified cellulose, edible gums, and copolymers. The dissolution rate of the film is controlled to facilitate incorporation of the medication into saliva or for absorption by the proximal mucosa. These films must be substantial enough to maintain their integrity during manufacture and packaging, and permit handling by the patient. Because of the rapid dissolution, taste and mouth feel are important considerations.

**Foams**

Foams are dispersions of gas in a liquid or solid continuous phase wherein the liquid or solid contains the drug substance and suitable excipients. Typical excipients intended for foam dosage forms include surfactants to ensure distribution of the gas/propellant in the formulation, aqueous or nonaqueous vehicles, and propellants (for pressurized systems). Foams are produced by mechanical means or via interaction of propellant gas and the formulation under pressure. Foams dispensed from nonpressurized containers use mechanical force to mix the formulation and air resulting in foam generation. Foams dispensed from pressurized containers use the propellant present in the gas phase to increase pressure inside the container. When the nozzle of the actuator is opened, the liquid phase is pushed out through specific actuators resulting in foam generation. Medicated foams intended to treat severely injured skin or open wounds must be sterile.

**PREPARATION**

A foam may contain one or more drug substances, surfactants, and aqueous or nonaqueous liquids, and is produced with or without the aid of propellants. When a propellant is not used, mechanical work is required to generate the foam. If the propellant is in the internal (discontinuous) phase, a stable foam is discharged. If the propellant is in the external (continuous) phase, a quick-breaking foam is discharged.

**Gases**

Medical gases are products that are administered directly as a gas. A medical gas has a direct pharmacological action or acts as a diluent for another medical gas. Gases used as excipients for administration of aerosol products, as adjuvants in packaging, or produced by other dosage forms, are not included in this definition.

**COMPONENTS**

Medical gases may be single components or defined mixtures of components. Mixtures can also be extemporaneously prepared at the point of use.

**ADMINISTRATION**

Medical gases may be administered to the patient using several methods: nasal cannulas, face masks, atmospheric tents, and endotracheal tubes for the pulmonary route; hyperbaric chambers for the pulmonary and topical routes of administration; jetted tubes that are directed at dental tissue to promote drying in preparation for fillings and crowns; tubes for expanding the intestines to facilitate medical imaging during colonoscopy; tubes for expanding the pelvis via transuterine inflation in preparation for fallopian tubal ligation; and tubes for expanding angioplasty devices. The dose of medical gas is typically metered by a volume rate of flow under ambient temperature and pressure conditions. Administration of a highly compressed gas generally requires a regulator to decrease the pressure, a variable-volume flow controller, and suitable tubing to conduct the
gas to the patient. For pulmonary administration, the gas flow will be directed to the nose or mouth by a suitable device or into the trachea through a mechanical ventilator. When medical gases are administered chronically, provision for humidification is common. Care should be exercised to avoid microbial contamination.

SPECIAL CONSIDERATIONS

The container and system fittings should be appropriate for the medical gas. Adaptors should not be used to connect containers to patient-use supply system piping or equipment. Large quantities of gases such as oxygen or nitrogen can be stored in the liquid state in a cryogenic container and converted into a gas, as needed, by evaporation. Additional rules concerning the construction and use of cryogenic containers are promulgated by governmental agencies (e.g., U.S. Department of Commerce).

Containers, tubing, and administration masks employed for gases containing oxygen are free of any compound that would be sensitive to oxidation or that would be irritating to the respiratory tract.

A significant fraction of the dose of a medical gas may be released into the general vicinity of the patient due to incomplete absorption. Adequate ventilation may be necessary to protect health care workers and others from exposure to the gas (e.g., nitrous oxide).

Gels

A gel is solid or semisolid. Gels can be classified in two groups, chemical and physical gels. Chemical gels are usually covalently crosslinked gels, while physical gels consist of small molecules or molecular chains that are physically crosslinked into networks, or solutions, or colloidal dispersions that are stiffened by a gelling agent. Typically, gels hold their form being self-supporting. Some gels may exhibit a range of behavior under mechanical forces. Gels may be thixotropic, forming semisolids on standing and becoming less viscous on agitation. Like emulsions, gels can be characterized as having a continuous phase as well as a dispersed phase. A variety of routes are available for gel administration such as topical, mucosal, or oral. In veterinary medicine, gels can also be administered via mammary infusion.

Gels may consist of a network of small discrete particles (e.g., Aluminum Hydroxide Gel, Bentonite Magma, or Psyllium Hemicellulose). As these gels may be thixotropic, forming semisolids on standing and becoming less viscous on agitation, they should be shaken before use to ensure homogeneity and should be so labeled.

Gels can consist of organic macromolecules uniformly distributed throughout a liquid in such a manner that no apparent boundaries exist between the dispersed macromolecules and the liquid continuous phase. These gels may be made from natural or synthetic macromolecules (e.g., carboxomer, hypromellose, or starch) or natural gums (e.g., tragacanth). Although these gels are commonly aqueous based, alcohols and oils may be used as the continuous phase.

Chewable gels are used to deliver drug substances or dietary supplements via the oral route. In addition to drug substances(s) or dietary supplements, chewable gels can consist of all or some of the following components—gelling agent(s), sugars, water, sweeteners, and flavoring agents. The sweeteners and flavoring are intended to enhance patient acceptance and mask the taste of the delivered labeled drug substance or dietary supplement. Chewable gels maintain their molded shape, are elastic, and yield to mastication. They are intended to be chewed before swallowing. Chewable gels are also known as “gummies” in the confectionary and dietary supplement industries but that term is not used in official article titles.

PREPARATION

Gels may be formed by dispersing the gelling agent in the continuous phase (e.g., by heating starch), by crosslinking the dispersed phase gelling agent, by changing the pH (as for Carbomer Copolymer), or by reducing the continuous phase by heat or vacuum (as for gels formed with sucrose).

Care should be taken to ensure uniformity of the drug substances by dispersing them by vigorous mixing or milling, or by shaking if the preparation is less viscous.

Chewable gels are formulated with one or more gelling agents (such as gelatin or starch), sugars (such as sucrose, fructose, or corn syrup), flavoring agents, sweeteners, colorants, and water. The ingredients are blended and heated to form a viscous solution that is poured into molds (e.g., corn starch molds). After cooling, the individual units are separated from the molds.

Granules

Granules are solid dosage forms that are composed of agglomerations of smaller particles. These multicomponent compositions are prepared for oral administration and are used to facilitate flexible dosing regimens as granules or as suspensions, address stability challenges, allow taste masking, or facilitate flexibility in administration (for instance, to pediatric patients, geriatric patients, or animals). Granular dosage forms may be formulated for direct oral administration and may facilitate compounding of multiple drug substances by allowing compounding pharmacists to blend various granular compositions in the retail or hospital pharmacy. More commonly, granules are reconstituted to a suspension by the addition of water or a supplied liquid diluent immediately prior to delivery to the patient. Effervescent granules are formulated to liberate gas (carbon dioxide) upon addition of water. Common examples of effervescent granules include antacid and potassium supplementation preparations. Common therapeutic classes formulated as granule dosage forms include antibiotics, certain laxatives (such as senna extract products), electrolytes, and a variety of cough and cold remedies that contain multiple drug substances.

PREPARATION

Granules are often the precursors used in tablet compression or capsule filling. Although this application represents a pharmaceutical intermediate and not a final dosage form, numerous commercial products are based on granules. In the typical
manufacture of granules, the drug substance(s) is blended with excipients (processing aids) and wetted with an appropriate pharmaceutical binding solution, solvent, or blend of solvents to promote agglomeration. This composition is dried and sized to yield the desired material properties.

Frequently, granules are used because the drug substance is unstable in aqueous environments and cannot be exposed to water for periods sufficient to accommodate manufacture, storage, and distribution in a suspension. Preparation of the liquid dosage form from the granules immediately prior to dispensing allows acceptable stability for the duration of use. Granules manufactured for this purpose are packaged in quantities sufficient for a limited time period—usually one course of therapy that typically does not exceed 2 weeks. In addition to the drug substances, other ingredients may be added to ensure acceptable stability (e.g., buffers, antioxidants, or chelating agents) or to provide color, sweetness, and flavor; and for suspensions, to provide acceptable viscosity to ensure adequate suspension of the particulate to enable uniform dosing.

Effervescent granules are typically formulated from sodium or potassium bicarbonate and an acid such as citric or tartaric acid. To prevent untimely generation of carbon dioxide, manufacturers should take special precautions to limit residual water in the product due to manufacture and to select packaging that protects the product from moisture. The manufacture of effervescent granules can require specialized facilities designed to maintain very low humidity (approximately 10% relative humidity). Effervescent powder mixtures are purposely formed into relatively coarse granules to reduce the rate of dissolution and provide a more controlled effervescence.

Reconstitution of granules must ensure complete wetting of all ingredients and sufficient time and agitation to allow the soluble components to dissolve. Specific instructions for reconstitution provided by the manufacturer should be carefully followed.

Reconstituted suspensions should be thoroughly mixed or shaken before use to resuspend the dispersed particulates. This is especially true of suspension preparations dosed from multiple-dose containers. For particularly viscous suspensions prone to air entrapment, instructions may advise the user how to shake the preparation to resuspend settled particulates while minimizing air entrapment.

For granules reconstituted to form suspensions for oral administration, acceptable suspension of the particulate phase depends on the particle size of the dispersed phase as well as the viscosity of the vehicle. Temperature can influence the viscosity, which influences suspension properties and the ease of removal of the dose from the bottles. In addition, temperature cycling can lead to changes in the particle size of the dispersed phase via Ostwald ripening. Thus, clear instructions should be provided regarding the appropriate storage temperature for the product.

**Gums**

Medicated gum is a pliable dosage form that is designed to be chewed rather than swallowed. Medicated gums release the drug substance(s) into the saliva. Medicated gums can deliver therapeutic agents for local action in the mouth or for systemic absorption via the buccal or gastrointestinal routes (e.g., nicotine or aspirin). Most gums are manufactured using the conventional melting process derived from the confectionery industry or alternatively may be directly compressed from gum powder. Medicated gums are formulated from insoluble synthetic gum bases such as polyisoprene, polyisobutylene, isobutylenesoprene copolymer, styrene butadiene rubber, polyvinyl acetate, polyethylene, ester gums, or polyterpenes. Plasticizers and softeners such as propylene glycol, glycerin, oleic acid, or processed vegetable oils are added to keep the gum base pliable and to aid in the incorporation of the drug substance(s), sweeteners, and flavoring agents. Sugars as well as artificial sweeteners and flavorings are incorporated to improve taste, and dyes may be used to enhance appearance. Some medicated gums are coated with magnesium stearate to reduce tackiness and improve handling during packaging. A preservative may be added.

**PREPARATION**

**Melted gum:** The gum base is melted at a temperature of about 115° until it has the viscosity of thick syrup and, at that point, is filtered through a fine-mesh screen. This molten gum base is transferred to mixing tanks where the sweeteners, plasticizers, and typically the drug substance are added and mixed. Colorings, flavorings, and preservatives are added and mixed while the melted gum is cooling. The cooled mixture is shaped by extrusion or rolling and cutting. Dosage units of the desired shape and potency are packaged individually. Additional coatings such as powder coatings to reduce tackiness or film or sugar coatings may be added to improve taste or facilitate bulk packaging.

**Directly compressed gum:** The gum base is supplied in a free-flowing granular powder form. The powder gum base is then dry blended with sweeteners, flavors, the drug substance, and lubricant. The blend is then processed through a conventional tablet press and tableted into desired shapes. The resulting medicated gum tablets can be further coated with sugar or sugar-free excipients. These tablets can be packaged in blisters or bottles as needed.

**SPECIAL CONSIDERATIONS**

Medicated gums are typically dispensed in unit-dose packaging. The patient instructions also may include a caution to avoid excessive heat.

**Implants**

Implants are long-acting dosage forms that provide continuous release of the drug substance often for periods of months to years. They are administered by the parenteral route and are sterile. Some implants approved as animal drugs to be administered subcutaneously to the ears are not required to be sterile. Typically for systemic delivery, they may be placed subcutaneously, or for local delivery they can be placed in a specific region in the body (e.g., in the sinus, in an artery, in the eye, in the brain, etc.). Implants are usually administered by means of a suitable injector or by surgical procedure.
Polymer implants can be formed as a single-shaped mass such as a cylinder. The polymer matrix must be biocompatible (see The Biocompatibility of Materials Used in Drug Containers, Medical Devices, and Implants (1031)), but it can be either bioabsorbable or nonbioabsorbable. Shaped polymer implants are administered by means of a suitable special injector. Release kinetics are typically not zero-order, but zero-order kinetics are possible. Drug substance release can be controlled by the diffusion of the drug substance from the bulk polymer matrix or by the properties of a rate-limiting polymeric membrane coating. Polymer implants are used to deliver potent small molecules like steroids (e.g., estradiol for cattle) and large molecules like peptides [e.g., luteinizing hormone-releasing hormone (LHRH)]. Example durations of drug substance release are 2 and 3 months for nonbioabsorbable implants and up to 3 years for bioabsorbable implants. An advantage of bioabsorbable implants is that they do not require removal after the release of all drug substance content. Nonbioabsorbable polymer implants can be removed before or after a drug substance release is complete or may be left in situ. An implant can have a tab with a hole in it to facilitate suturing it in place (e.g., for an intravitreal implant for local ocular delivery). Such implants may provide therapeutic release for periods as long as 2.5 years.

Drug substance-eluting stents combine the mechanical effect of the stent to maintain arterial patency with the prolonged pharmacologic effect of the incorporated drug substance (to reduce restenosis, inhibit clot formation, or combat infection). As an example, a metal stent can be coated with a nonbioabsorbable or bioabsorbable polymer-containing drug substance. The resultant coating is a polymeric matrix that controls the extended release of the drug substance.

PREPARATION

Cylindrical polymeric implants are commonly made by melt extrusion of a blend of drug substance and polymer, resulting in a rod that is cut into shorter lengths. Polymer implants can also be made by injection molding. Still other implants are assembled from metal tubes and injection-molded plastic components.

Sterility can be achieved by terminal sterilization or by employing aseptic manufacturing procedures.

Injections

(See Emulsions, Powders, Solutions, and Suspensions.)

Injections are not treated as a dosage form in this chapter. Chapter (1) provides quality and other information about injectable products. Information on specific dosage form terminology can be found in the Glossary. For appropriate injection nomenclature, see Nomenclature (1121).

EXCESS VOLUME IN INJECTIONS

Each container of an injection is filled with a volume in slight excess of the labeled “size” or the volume that is to be withdrawn. The excess volumes recommended in Table 1 are usually sufficient to permit withdrawal and administration of the labeled volumes.

Table 1

<table>
<thead>
<tr>
<th>Labeled Size (mL)</th>
<th>Recommended Excess Volume</th>
<th>For Mobile Liquids (mL)</th>
<th>For Viscous Liquids (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td></td>
<td>0.10</td>
<td>0.12</td>
</tr>
<tr>
<td>1.0</td>
<td></td>
<td>0.10</td>
<td>0.15</td>
</tr>
<tr>
<td>2.0</td>
<td></td>
<td>0.15</td>
<td>0.25</td>
</tr>
<tr>
<td>5.0</td>
<td></td>
<td>0.30</td>
<td>0.50</td>
</tr>
<tr>
<td>10.0</td>
<td></td>
<td>0.50</td>
<td>0.70</td>
</tr>
<tr>
<td>20.0</td>
<td></td>
<td>0.60</td>
<td>0.90</td>
</tr>
<tr>
<td>30.0</td>
<td></td>
<td>0.80</td>
<td>1.20</td>
</tr>
<tr>
<td>50.0 or more</td>
<td></td>
<td>2%</td>
<td>3%</td>
</tr>
</tbody>
</table>

Inserts

Inserts are solid dosage forms that are inserted into a naturally occurring (nonsurgical) body cavity other than the mouth or rectum (see Suppositories). The drug substance in inserts is delivered for local or systemic action. Vaginal inserts are usually globular or oviform and weigh about 5 g each. Inserts intended to dissolve in vaginal secretions are usually made from water-soluble or water-miscible vehicles such as polyethylene glycol or glycerinated gelatin.

PREPARATION

For general considerations, see (795). Inserts vary considerably in their preparation. Inserts may be molded (using technology similar to that used to prepare lozenges, suppositories, or plastics), compressed from powders (as in tableting), or formulated as special applications of capsules (soft gelatin capsules and hard gelatin capsules have been employed for extemporaneously compounded preparations). Inserts may be formulated to melt at body temperature or disintegrate upon insertion. Design of...
the dosage form should take into consideration the fluid volume available at the insertion site and minimize the potential to cause local irritation. Most inserts are formulated to ensure retention at the site of administration.

**Irrigations**

(See *Solutions*.)

**Liquids**

As a dosage form, a liquid consists of a pure chemical in its liquid state. Examples include mineral oil, isoflurane, and ether. This dosage form term is not applied to solutions.

**Lotions**

(See *Emulsions*.)

**Lozenges**

Lozenges are solid oral dosage forms that are designed to dissolve or disintegrate slowly in the mouth. They contain one or more drug substances that are slowly liberated from the, typically, flavored and sweetened base. They are frequently intended to provide local action in the oral cavity or the throat but also include those intended for systemic absorption after dissolution. The typical therapeutic categories of drug substances delivered in lozenges are antiseptics, analgesics, decongestants, antitussives, and antibiotics. Molded lozenges are called cough drops or pastilles but these terms are not used in official article titles. Lozenges prepared by compression or by stamping or cutting from a uniform bed of paste are sometimes known as troches (a term not used in official article titles). Compressed or stamped lozenges are often produced in a circular shape.

Lozenges can be made using sugars such as sucrose and dextrose, or can provide the benefits of a sugar-free formulation that is usually based on sorbitol or mannitol. Polyethylene glycols and hypromellose are sometimes included to slow the rate of dissolution.

**PREPARATION**

Excipients used in molded lozenge manufacture include gelatin, fused sucrose, sorbitol, or another carbohydrate base. Molded lozenges can be prepared by mixing the ingredients with water and heating to reduce the water content. The viscous solution is then poured into molds (e.g., corn starch molds). The lozenges are quickly cooled in the molds to trap the base in the glassy state. Once formed, the lozenges are removed from the molds and packaged. Care is taken to avoid excessive moisture during storage to prevent crystallization of the sugar base.

Compressed lozenges are made using excipients that may include a filler, binder, sweetening agent, flavoring agent, and lubricant. Sugars such as sucrose, sorbitol, and mannitol are often included because they can act as a filler and binder as well as serve as sweetening agents. Approved FD&C and D&C dyes or lakes (dyes adsorbed onto insoluble aluminum hydroxide) may also be present.

The manufacturing of compressed lozenges is essentially the same as that for conventional tableting, with the exception that a tablet press capable of making larger tablets and exerting greater force to produce harder tablets may be required (see *Tablets*).

The paste used to produce lozenges manufactured by stamping or cutting contains a moistening agent, sucrose, and flavoring and sweetening agents. The homogenous paste is spread as a bed of uniform thickness, and the lozenges are cut or stamped from the bed and are allowed to dry. Some lozenges are prepared by forcing dampened powders under low pressure into mold cavities and then ejecting them onto suitable trays for drying at moderate temperatures.

**Ointments**

Ointments are semisolid preparations generally intended for external application to the skin or mucous membranes. Drug substances delivered in ointments are intended for local action or for systemic absorption. Ointments usually contain less than 20% water and volatiles, and more than 50% hydrocarbons, waxes, or polyols as the vehicle. Ointment bases recognized for use as vehicles fall into four general classes: hydrocarbon bases, absorption bases, water-removable bases, and water-soluble bases.

**HYDROCARBON BASES**

Also known as oleaginous ointment bases, hydrocarbon bases allow the incorporation of only small amounts of an aqueous component. Ointments prepared from hydrocarbon bases act as occlusive dressings and provide prolonged contact of the drug substance with the skin. They are difficult to remove and do not change physical characteristics upon aging.

**ABSORPTION BASES**

Absorption bases allow the incorporation of aqueous solutions. Such bases include only anhydrous components (e.g., *Hydroporphic Petrolatum*) or W/O emulsions (e.g., *Lanolin*). Absorption bases are also useful as emollients.
WATER-REMOVABLE BASES

O/W emulsions (e.g., Hydrophilic Ointment) are sometimes referred to as creams (see Emulsions). Water-removable bases may be readily washed from the skin or clothing with water, making them acceptable for cosmetic reasons. Other advantages of the water-removable bases are that they can be diluted with water and that they favor the absorption of serous discharges in dermatological conditions.

WATER-SOLUBLE BASES

Also known as greaseless ointment bases, they are formulated entirely from water-soluble constituents. Polyethylene Glycol Ointment is the only official preparation in this group. Water-soluble bases offer many of the advantages of the water-removable bases and, in addition, contain no water-insoluble substances such as petrolatum, anhydrous lanolin, or waxes. They are more correctly categorized as gels (see Gels).

The choice of an ointment base depends on the action desired, the characteristics of the incorporated drug substance, and the latter’s bioavailability if systemic action is desired. The product’s stability may require the use of a base that is less than ideal in meeting other quality attributes. Drug substances that hydrolyze rapidly, for example, are more stable in hydrocarbon bases than in bases that contain water.

PREPARATION

Ointments are typically prepared by either direct incorporation into a previously prepared ointment base or by fusion (heating during the preparation of the ointment). A levigating agent is often added to facilitate the incorporation of the medicament into the ointment base by the direct incorporation procedure. In the fusion method, the ingredients are heated. Homogenization is often necessary. The rate of cooling is an important manufacturing detail because rapid cooling can impart increased structure to the product of the fusion method.

Pastes

Pastes are semisolid preparations of stiff consistency and contain a high percentage (20%–50%) of finely dispersed solids. Pastes are intended for application to the skin, oral cavity, or mucous membranes. Pastes ordinarily do not flow at body temperature and thus can serve as occlusive, protective coatings. As a consequence, pastes are more often used for protective action than as ointments.

Fatty pastes that have a high proportion of hydrophilic solids appear less greasy and are more absorptive than ointments. They are used to absorb serous secretions and are often preferred for acute lesions that have a tendency toward crusting, vesiculation, or oozing.

Dental pastes are applied to the teeth. Other orally administered pastes may be indicated for adhesion to the mucous membrane for a local effect.

In veterinary medicine, pastes are typically administered orally and are intended for systemic delivery of drug substances. The paste is squeezed into the mouth of the animal, generally at the back of the tongue, or is spread inside the mouth.

Pellets

The use of the term “pellet” for implantable dosage forms is no longer preferred (see Implants). In veterinary medicine, medicated articles and feeds may be pelletized but are not considered dosage forms (see Animal Drugs For Use In Animal Feeds (1152)).

Pellets are small solid dosage forms that can be designed as single or multiple entities. They can have a spherical or nearly spherical shape, although such a shape is not required. Spherical pellets are sometimes referred to as beads. Pellets used in veterinary medicine may instead be cylindrical in shape. Pellets can provide several advantages, including physical separation for chemically or physically incompatible materials and for control of the release of drug substance. Pellets may be designed with the drug substance dispersed in a matrix or the pellets may be coated with an appropriate polymer. Pellets may be administered by the oral (gastrointestinal) route. Pellets for oral administration can:

1. Protect stomach tissues from irritation
2. Sometimes minimize variability associated with gastric retention of larger dosage forms
3. Solely extend the release of the drug substance
4. Solely delay the release
5. Both extend and delay the release of the drug substance

Some pellets can be sprinkled on food. In the case of delayed-release formulations, the coating polymer is chosen to resist dissolution at the lower pH of the gastric environment but to dissolve in the higher pH of the intestinal environment.

Pellets may be administered by injection. One or several pellets can be injected or surgically administered to provide continuous therapy for periods of months or years (see Implants).

In veterinary medicine, pellets may be used to improve palatability of the drug product and pellets for oral administration may be delivered on top of an individual animal’s food or feed.

PREPARATION

The desired performance characteristics determine the manufacturing method chosen. In general, pellet dosage forms are manufactured by compression, or by wet or dry extrusion processes sometimes followed by spheronization, or followed by wet or dry coating processes. Manufacture of pellets by wet coating usually involves the application of successive coatings upon
nonpareil seeds. This manufacturing process is frequently conducted in fluid-bed processing equipment. Dry powder coating or layering processes are often performed in specialized rotor granulation equipment. The extent of particle growth achievable in wet coating processes is generally more limited than the growth that can be obtained with dry powder layering techniques, but either method allows the formulator to develop and apply multiple layers of coatings to achieve the desired release profile. The manufacture of pellets by compression is largely restricted to the production of material for subcutaneous implantation. This method of manufacture provides the necessary control to ensure dose uniformity and is generally better suited to aseptic processing requirements.

Alternatively, microencapsulation techniques can be used to manufacture pellets. Coacervation coating techniques typically produce coated particles that are much smaller than those made by other techniques.

Pills

Pills are drug substance-containing small, spherical, solid bodies intended for oral administration. The pill dosage form has been largely replaced by compressed tablets and by capsules. Unlike tablets, pills are usually prepared by a wet massing, piping, and molding technique. This term is frequently incorrectly used as a general term to describe solid oral dosage forms, such as tablets and capsules.

PREPARATION

Excipients are selected on the basis of their ability to produce a mass that is firm and plastic. The drug substance is triturated with powdered excipients in serial dilutions to attain a uniform mixture. Liquid excipients that act to bind and provide plasticity to the mass are subsequently added to the dry materials. The mass is formed by kneading. The properties of firmness and plasticity are necessary to permit the mass to be worked and retain the shape produced. Cylindrical pill pipes are produced from portions of the mass. The pill pipe is cut into individual lengths corresponding to the intended pill size, and the pills are rolled to form the final shape. Pill-making machines can automate the preparation of the mass, production of pill piping, and the cutting and rolling of pills.

Plasters

A plaster is a semisolid substance for external application that is supplied on a support material. Plasters are applied for prolonged periods to provide protection, support, or occlusion (maceration). This term is not preferred and should not be used for new drug product titles. Plasters consist of an adhesive layer that may contain active substances. This layer is spread uniformly on an appropriate support that is usually made of a rubber base or synthetic resin. Unmedicated plasters are designed to provide protection or mechanical support to the site of application. Plasters are available in a range of sizes or cut to size to effectively provide prolonged contact to the site of application. They adhere firmly to the skin but can be peeled off the skin without causing injury.

Powders

Powders are defined as a single solid or a mixture of solids in a finely divided state. Powders used as pharmaceutical dosage forms may contain one or more drug substances and can be used as is or can be mixed with a suitable vehicle for administration. (See Solutions or Suspensions.) Powders can be intended for internal or external use. Powders for external use are typically dusted onto the skin or applied to bandages or clothing. Powders for internal use can be applied to accessible mucous membranes with suitable applicators or are entrained in air streams for application to the nose or lungs.

The performance of powder dosage forms can be affected by the physical characteristics of the powder. Selection of relevant and appropriate powder characteristics depends on the dosage form and its route of administration. For example, particle size can influence the dissolution rate of the particles and thus the bioavailability and/or effectiveness at the site of action. Externally applied powders should have a particle size of 150 μm or less (typically in the 50- to 100-μm range to prevent a gritty feel on the skin that could further irritate traumatized skin). The particle size of powders delivered to the lung or nose influences where the powder is deposited. Particle size may influence the mixing, segregation, and aggregation of the particles, which can affect the delivery and uniformity of the dosage form. For more information, see Powder Finesness (811) and (5).

In veterinary medicine, a powder that needs to be reconstituted prior to administration has been called a concentrate (e.g., drug products administered via drinking water). Such use of the term “concentrate” is no longer preferred.

INHALATION POWDERS AND NASAL POWDERS

Inhalation powders and nasal powders consist of an appropriately finely divided solid and a suitable container–closure delivery system. For additional information, see (5) and (601).

PREPARATION

Powder dosage forms can be produced by the combination of multiple components into a uniform blend. This preparation can also involve particle size reduction, a process referred to as comminution. Milling, spray drying, supercritical fluid, high-pressure homogenization, precipitation technologies, and porous microparticle fabrication techniques may be used to reduce the particle size of powders. As the particle size is decreased, the number of particles and the surface area increase, which can increase the dissolution rate and bioavailability, and/or the rate and extent of local action, of the drug substance.

Blending of powders may be accomplished by different techniques. Industrial processes may employ sifting or tumbling the powders in a rotating container. One of the most common tumble blenders is a V-blender, which is available in a variety of
sizes suitable for small-scale and large-scale compounding and industrial production. Depending on the particle size of the drug substance, a random mixture of powders may be employed. Blending techniques for powders include those used in compounding pharmacy such as spatulation and trituration (see \textit{substance}).

Powder flow can be influenced by both particle size and shape. Larger particles generally flow more freely than do fine particles. Powder flow is an important attribute that can affect the packaging or dispensing of a powder.

**Rinses**

(See \textit{Solutions}.)

**Soaps and Shampoos**

Soaps and shampoos are solid or liquid preparations intended for topical application to the skin or scalp followed by subsequent rinsing with water. Soaps and shampoos are emulsions, suspensions, or surface-active compositions that readily form emulsions, micelles, or foams upon the addition of water followed by rubbing. Incorporation of drug substances in soaps and shampoos combines the cleansing/degreasing abilities of the vehicle and facilitates the topical application of the drug substance to affected areas, even large areas, of the body. The surface-active properties of the vehicle facilitate contact of the drug substance with the skin or scalp. Medicated soap and shampoo formulations frequently contain suitable antimicrobial agents to protect against bacteria, yeast, and mold contamination.

**PREPARATION**

The preparation of medicated soaps and shampoos follows techniques frequently used for the preparation of emulsified systems. To ensure uniformity, the drug substance(s) must be added to the vehicle prior to congealing (in the case of soaps) followed by thorough mixing. If the medication is present as a suspension, the particle size must be controlled to promote uniform distribution of the drug substance and possibly optimize performance. Because soap manufacture frequently involves processing the ingredients at an elevated temperature, care must be exercised to avoid excessive degradation of the drug substance during processing.

**Solutions**

A solution is a preparation that contains one or more dissolved chemical substances in a suitable solvent or mixture of mutually miscible solvents. Because molecules of a drug substance in solution are uniformly dispersed, the use of solutions as dosage forms generally provides assurance of uniform dosage upon administration and good accuracy when the solution is diluted or otherwise mixed.

Substances in solutions are more susceptible to chemical instability than they are in the solid state and, dose-for-dose, are generally heavier and more bulky than solid dosage forms. These factors increase the cost of packaging and shipping relative to that of solid dosage forms. Some solutions are prepared and ready for use, and others are prepared as powders or other solids intended for reconstitution with an appropriate vehicle just before use (see \textit{Powders}). Solution dosage forms can be administered by injection, inhalation, and the mucosal, topical, and gastrointestinal routes. A solution administered by injection is officially titled “injection” (see (1)).

Some solutions are designed to form a mass in situ. These solutions comprise polymer, drug substance, and solvent for the polymer. The polymer solvent can be water or an organic solvent. After administration of the solution to a patient by subcutaneous or intramuscular administration, it forms a gel or a solid polymeric matrix that traps the drug substance and extends the drug substance release for days or months.

Solutions intended for oral administration usually contain flavorings and colorants to make the medication more attractive and palatable for the patient or consumer. When needed, they also may contain stabilizers to maintain chemical and physical stability and preservatives to prevent microbial growth.

Solutions are sometimes placed on devices such as swabs, cloths, or sponges, that aid application.

In veterinary medicine, a solution that needs to be diluted prior to administration has been called a concentrate (e.g., drug products administered via drinking water). Such use of the term “concentrate” is no longer preferred.

**Sprays**

Spray preparations may deliver either accurately metered or nonmetered amounts of formulation. A spray drug product is a dosage form that contains a drug substance in the liquid state as a solution or suspension and is intended for administration as a mist. Sprays are distinguished from aerosols in that spray containers are not pressurized. Most of the sprays are generated by manually squeezing a flexible container or actuation of a pump that generates the mist by discharging the contents through a nozzle.

Depending on the design of the formulation and the valve system, the droplets generated may be intended for immediate inhalation through the mouth and deposition in the pulmonary tree, or for inhalation into the nose and deposition in the nasal cavity.

The mechanism for droplet generation and the intended use of the preparation distinguish various classes of sprays. A spray may be composed of a pump, container, actuator, valve, nozzle, or mouthpiece in addition to the formulation containing the drug(s), solvent(s), and any excipient(s). The design of each component plays a role for the appropriate performance of the drug product and in determining the critical characteristics of the droplet size distribution. Droplet and particle size distributions, delivered dose uniformity, plume geometry, and droplet velocity are critical parameters that influence the efficiency of drug delivery. When the preparation is supplied as a multidose container, the addition of a suitable antimicrobial preservative may
be necessary. Spray formulations intended for local or systemic effect typically have an aqueous base and may contain excipients to control pH and viscosity. In addition, depending on the route of administration, the formulation may be isotonic. For additional information, see (5) and (601).

LABELING AND USE


Strips

A strip is a dosage form or device in the shape of a long, narrow, thin, absorbent, solid material such as filter paper. Typically it is sterile and it may be impregnated with a compound or be gauged to allow measurements for diagnostic purposes, such as in measuring tear production. The term “strip” should not be used when another term such as “film” is more appropriate.

Suppositories

Suppositories are dosage forms adapted for application into the rectum. They melt, soften, or dissolve at body temperature. A suppository may have a local protectant or palliative effect, or may deliver a drug substance for systemic or local action.

Suppository bases typically include cocoa butter, glycerinated gelatin, hydrogenated vegetable oils, mixtures of polyethylene glycols of various molecular weights, and fatty acid esters of polyethylene glycol. The suppository base can have a notable influence on the release of the drug substance(s). Although cocoa butter melts quickly at body temperature, it is immiscible with body fluids and this inhibits the diffusion of fat-soluble drug substances to the affected sites. Polyethylene glycol is a suitable base for some antiseptics. In cases when systemic action is desired, incorporating the ionized rather than the nonionized form of the drug substance may help maximize bioavailability. Although nonionized drug substances partition more readily out of water-miscible bases such as glycerinated gelatin and polyethylene glycol, the bases themselves tend to dissolve very slowly, which slows drug substance release. Cocoa butter and its substitutes (e.g., Hard Fat) perform better than other bases for allaying irritation in preparations intended for treating internal hemorrhoids. Suppositories for adults are tapered at one or both ends and usually weigh about 2 g each.

PREPARATION

Cocoa butter suppositories have cocoa butter as the base and can be made by incorporating the finely divided drug substance into the solid oil at room temperature and suitably shaping the resulting mass, or by working with the oil in the melted state and allowing the resulting suspension to cool in molds. A suitable quantity of hardening agents may be added to counteract the tendency of some drug substances (such as chloral hydrate and phenol) to soften the base. The finished suppository melts at body temperature.

A variety of vegetable oils, such as coconut or palm kernel, modified by esterification, hydrogenation, or fractionation, are used as cocoa butter substitutes to obtain products that display varying compositions and melting temperatures (e.g., Hydrogenated Vegetable Oil and Hard Fat). These products can be designed to reduce rancidity while incorporating desired characteristics such as narrow intervals between melting and solidification temperatures, and melting ranges to accommodate formulation and climatic conditions.

Drug substances can be incorporated into glycerinated gelatin bases by addition of the prescribed quantities to a vehicle consisting of about 70 parts of glycerin, 20 parts of gelatin, and 10 parts of water.

Several combinations of polyethylene glycols that have melting temperatures that are above body temperature are used as suppository bases. Because release from these bases depends on dissolution rather than on melting, there are significantly fewer problems in preparation and storage than is the case for melting-type vehicles. However, high concentrations of higher molecular weight polyethylene glycols may lengthen dissolution time, resulting in problems with retention.

Several nonionic surface-active agents closely related chemically to the polyethylene glycols can be used as suppository vehicles. Examples include polyoxyethylene sorbitan fatty acid esters and the polyoxyethylene stearates. These surfactants are used alone or in combination with other suppository vehicles to yield a wide range of melting temperatures and consistencies. A notable advantage of such vehicles is their water dispersibility. However, care must be taken with the use of surfactants because they may either increase the rate of drug substance absorption or interact with the drug substance to reduce therapeutic activity.

Compounding suppositories using a suppository base typically involves melting the suppository base and dissolution or dispersion of the drug substance in the molten base (see (795)). When compounding suppositories, the compounding professional prepares an excess amount of total formulation to allow the prescribed quantity to be accurately dispensed. In compounding suppositories, avoid caustic or irritating ingredients, carefully select a base that will allow the drug substance to provide the intended effect, and in order to minimize abrasion of the rectal membranes, reduce solid ingredients to the smallest reasonable particle size.

Suspensions

A suspension is a biphasic preparation consisting of solid particles dispersed throughout a liquid phase. Suspension dosage forms may be formulated for specific routes of administration such as oral, topical, inhalation, ophthalmic, otic, and injection. Some suspensions are prepared and ready for use, and others are prepared as powders or other solids intended for reconstitution with an appropriate vehicle just before use (see Powders).
Inhalation suspensions (see (5)), ophthalmic suspensions, injectable suspensions, and some otic suspensions are prepared in sterile form. Suspensions are generally not injected intravenously, epidurally, or intratheceal unless the product labeling clearly specifies these routes of administration.

Some liposomal drug products are referred to as suspensions because they can settle and require resuspension prior to administration (see (1)).

Resorbable microparticles can provide extended release of a drug substance over periods varying from a few weeks to months. They can be administered subcutaneously or intramuscularly for systemic delivery, or they may be deposited in a desired location in the body for site-specific delivery. Resorbable microparticles (or microspheres) generally range from 20 to 100 µm in diameter. They are composed of a drug substance dispersed within a biocompatible, bioabsorbable polymeric excipient (matrix).

Poly(lactide-co-glycolide) polymers have been used frequently. These excipients typically resorb by hydrolysis of ester linkages. The microparticles are typically administered by suspension in an aqueous vehicle followed by injection with a conventional syringe and needle. Release of the drug substance from the microparticles begins after physiological fluid enters the polymer matrix, dissolving some of the drug substance that is then released by a diffusion-controlled process. Drug release also can occur as the bioresorbable polymer molecular weight decreases and as the matrix erodes.

Some suspensions are designed to form a mass in situ. These suspensions comprise polymer, drug substance, and solvent for the polymer. The polymer solvent can be water or an organic solvent. After administration of the suspension to a patient by subcutaneous or intramuscular administration, it forms a gel or a solid polymeric matrix that traps the drug substance and extends the drug substance release for days or months.

Historically, the term “milk” was sometimes used for suspensions in aqueous vehicles intended for oral administration (e.g., Milk of Magnesia). The term “magma” is often used to describe suspensions of inorganic solids, such as clays in water, that display a tendency toward strong hydration and aggregation of the solid, giving rise to gel-like consistency and thixotropic rheological behavior (e.g., Bentonite Magma). In the past, the term “lotion” referred to both topical suspensions and topical emulsions. Now the term only refers to topical emulsions (see Emulsions).

Limited aqueous solubility of the drug substance(s) is the most common rationale for developing a suspension. Other potential advantages of an oral suspension include taste masking and improved patient compliance because of the more convenient dosage form. When compared to solutions, suspensions can have improved chemical stability. Ideally, a suspension should contain small uniform particles that are readily suspended and easily redispersed following settling. Unless the dispersed solid is colloidal, the particulate matter in a suspension will likely settle to the bottom of the container upon standing. Such sedimentation may lead to caking and solidification of the sediment and difficulty in redispersing the suspension upon agitation. To prevent such problems, manufacturers commonly add ingredients to increase viscosity and the gel state of the suspension or flocculation, including clays, surfactants, polyols, polymers, or sugars. Frequently, thixotropic vehicles are used to counter particle-settling tendencies, but these vehicles must not interfere with pouring or redispersal. Additionally, the density of the dispersed phase and continuous phase may be modified to further control settling rate. For topical suspensions, rapid drying upon application is desirable.

Temperature can influence the viscosity (and thus suspension properties and the ease of removing the dose from the bottle), and temperature cycling can lead to changes in the particle size of the dispersed phase via Ostwald ripening. When manufacturers conduct stability studies to establish product shelf life and storage conditions, they should cycle conditions (freeze/thaw) to investigate temperature effects.

In veterinary medicine, a suspension that needs to be diluted prior to administration has been called a concentrate (e.g., drug products administered via drinking water). Such use of the term “concentrate” is no longer preferred.

**PREPARATION**

Suspensions are prepared by adding suspending agents or other excipients and purified water or oil to solid drug substances and mixing to achieve uniformity. In the preparation of a suspension, the characteristics of both the dispersed phase and the dispersion medium should be considered. During development, manufacturers should define an appropriate particle size distribution for the suspended material to achieve the desired effectiveness and to minimize the likelihood of particle size changes during storage.

In some instances, the dispersed phase has an affinity for the vehicle and is readily wetted upon its addition. For some materials, the displacement of air from the solid surface is difficult, and the solid particles may clump together or float on top of the vehicle. In the latter case, a wetting agent may be used for certain types of suspensions to facilitate displacement of air from the powder surface. Surfactants, alcohol, glycerin, and other hydrophilic surfactants can be used as wetting agents when an aqueous vehicle will be used as the dispersion phase. These agents function by displacing the air in the crevices of the particles and dispersing the particles. In the large-scale preparation of suspensions, wetting of the dispersed phase may be aided by the use of high-energy mixing equipment such as colloid mills or other rotor–stator mixing devices.
After the powder has been wetted, the dispersion medium (containing the soluble formulation components such as colorants, flavorings, and preservatives) is added in portions to the powder, and the mixture is thoroughly blended before subsequent additions of the vehicle. A portion of the vehicle is used to wash the mixing equipment free of suspended material, and this portion is used to bring the suspension to final volume and ensure that the suspension contains the desired concentration of solid matter. The final product may be passed through a colloid mill or other blender or mixing device to ensure uniformity.

Suspensions are resuspended before the dose is dispensed. Because of the viscosity of many suspension vehicles, air entrainment may occur during dosing. The formulation process allows evaluation of this possibility; adjustments in vehicle viscosity or the incorporation of low levels of antifoaming agents are common approaches to minimize air entrainment. Alternatively, specific instructions for resuspending the formulation may be provided to minimize air incorporation and ensure accurate dosing.

Systems

Systems are preparations of drug substance(s) in carrier devices, often containing adhesive backing, that are applied topically or inserted into body cavities. The drug substance is designed to be released in a controlled manner over a specified period of time or the drug substance is released based on its concentration in the formulation. Unless otherwise stated in the labeling, the carrier device is removed after use. The term “system” should not be used when another dosage form term is more appropriate (e.g., inserts and implants).

The notation of strength is either defined in terms of the amount of the drug substance released from the system over a specific period of time or as the drug concentration within the formulation (e.g., the percentage of the drug). Various routes of administration are possible, so the route must always be indicated in the compendial name when a specific location for application is essential for proper use (e.g., “intrauterine”, “ocular”, or “periodontal” as the route of administration). For example, systems applied to the eye are called oculum systems. The route is named “transdermal” when, for example, systemic absorption of the drug substance may take place through the dermis without specifying the region of the body to which the system is applied.

The term “patch” has sometimes been used but is not preferred for use in drug product monograph nomenclature when referring to a system.

Intrauterine systems are intended for placement in the uterus. Release of the drug substance can be up to 5 years.

Ocular systems are intended for placement in the lower conjunctival fornix from which the drug diffuses through a membrane at a constant rate.

Periodontal systems are intended for placement in the pocket between the tooth and the gum. In some cases, periodontal systems may be formed in situ in the periodontal pocket and release the drug substance(s) for several weeks.

Transdermal systems (TDS) are placed onto intact skin to deliver the drug to the systemic circulation. They are designed for prolonged release (up to 7 days). Specific quality tests for TDS are found in (3).

Tablets

Tablets are solid dosage forms in which the drug substance is generally blended with excipients and compressed into the final dosage. Tablets are the most widely used dosage form in the United States. Tablet presses use steel punches and dies to prepare compacted tablets by the application of high pressures to powder blends or granulations. Tablets can be produced in a wide variety of sizes, shapes, and surface markings. Capsule-shaped tablets are commonly referred to as caplets, although the term is not used in official article titles. Specialized tablet presses may be used to produce tablets with multiple layers or with specially formulated core tablets placed in the interior of the final dosage form. These specialized tablet presentations can delay or extend the release of the drug substance(s) or physically separate incompatible drug substances. Tablets may be coated by a variety of techniques to provide taste masking, protection of photo-labile drug substance(s), extended or delayed release, or unique appearance (colors). When no deliberate effort has been made to modify the drug substance release rate, tablets are referred to as immediate-release.

BUCCAL TABLETS

Intended to be inserted in the buccal pouch, where the drug substance is absorbed directly through the oral mucosa. Few drug substances are readily absorbed in this way (examples are nitroglycerin and certain steroid hormones).

CHEWABLE TABLETS

Formulated and manufactured to produce a pleasant-tasting residue in the mouth and to facilitate swallowing. Hard chewable tablets are typically prepared by compaction, usually utilizing mannitol, sorbitol, or sucrose as binders and fillers, and contain colors and flavors to enhance their appearance and taste. Soft chewable tablets are typically made by a molding or extrusion process, frequently with more than 10% water to help maintain a pliable, soft product. Hard chewable tablets in veterinary medicine often have flavor enhancers like brewer’s yeast or meat/fish-based flavors.

Tablets for human use that include “chewable” in the title must be chewed or crushed prior to swallowing to ensure reliable release of the drug substance(s) or to facilitate swallowing. If tablets are designed so that they may be chewed (but chewing is not required for drug substance release or ease of swallowing), the title should not include a reference to “chewable”. In that case, the product may still be described as “chewable” in the ancillary labeling statement.

Tablets for veterinary use that are intended to be chewed will include “Chewable” in the title. However, it is understood that for veterinary products it is not possible to ensure that tablets are chewed prior to ingestion. Chewable tablets may be broken into pieces and fed to animals that normally swallow treats whole.
EFFERVESCENT TABLETS

Prepared by compaction and contain, in addition to the drug substance(s), mixtures of acids (e.g., citric acid or tartaric acid) and carbonates, and/or sodium bicarbonate. Upon contact with water, these formulations release carbon dioxide, producing the characteristic effervescent action.

HYPODERMIC TABLETS

Molded tablets made from completely and readily water-soluble ingredients; formerly intended for use in making preparations for hypodermic injection. They may be administered orally or sublingually when rapid drug substance availability is required.

MODIFIED-RELEASE TABLETS

There are two categories of modified-release tablet formulations recognized by USP.

Delayed-release tablets: Tablets are sometimes formulated with acid-resistant or enteric (also called “gastro-resistant”) coatings to protect acid-labile drug substances from the gastric environment or to prevent adverse events such as irritation.

Extended-release tablets: Extended-release tablets are formulated in such a manner as to make the drug substance available over an extended period of time following ingestion. Requirements for dissolution (see 711) are typically specified in the individual monographs.

ORALLY DISINTEGRATING TABLETS

Orally disintegrating tablets are intended to disintegrate rapidly within the mouth to provide a dispersion before the patient swallows the resulting slurry where the drug substance is intended for gastrointestinal delivery and/or absorption. Some of these dosage forms have been formulated to facilitate rapid disintegration and are manufactured by conventional means or by using lyophilization or molding processes. Further details may be found in the CDER Guidance for Industry: Orally Disintegrating Tablets.

SUBLINGUAL TABLETS

Sublingual tablets are intended to be inserted beneath the tongue, where the drug substance is absorbed directly through the oral mucosa. As with buccal tablets, few drug substances are extensively absorbed in this way, and much of the drug substance is swallowed and is available for gastrointestinal absorption.

TABLETS FOR ORAL SOLUTION

Before administration, tablets for oral solution are intended to be solubilized in a liquid diluent. In some cases, tablets for oral solution may also be chewed or swallowed.

TABLETS FOR ORAL SUSPENSION

Tablets for oral suspension are intended to be dispersed in a liquid before administration as a suspension. The dosage form is tablets for oral suspension when either the drug substance or the excipients do not dissolve when dispersed in a liquid. In some cases, tablets for oral suspension may also be chewed or swallowed.

TABLET TRITURATES

Small, usually cylindrical, molded or compacted tablets. Tablet triturates traditionally were used as dispensing tablets in order to provide a convenient, measured quantity of a potent drug substance for compounding purposes, but they are rarely used today.

PREPARATION

Most compacted (compressed) tablets consist of the drug substance(s) and a number of excipients. These excipients may include fillers (diluents), binders, disintegrating agents, lubricants, and glidants. Approved FD&C and D&C dyes or lakes, flavors, and sweetening agents may also be present.

Fillers or diluents are added when the quantity of drug substance(s) is too small or the properties of the drug substance do not allow satisfactory compaction in the absence of other ingredients. Binders impart adhesiveness to the powder blend and promote tablet formation and maintenance of drug substance uniformity in the tableting mixture. Disintegrating agents facilitate reduction of the tablet into small particles upon contact with water or biological fluids. Lubricants reduce friction during the compaction and ejection cycles. Glidants improve powder fluidity, powder handling properties, and tablet weight control. Colorants are often added to tablet formulations for aesthetic value or for product identification.

Tablets are prepared from formulations that have been processed by one of three general methods: wet granulation, dry granulation (roll compaction or slugging), and direct compression.

Wet granulation: Involves the mixing of dry powders with a granulating liquid to form a moist granular mass that is dried and sized prior to compression. It is particularly useful in achieving uniform blends of low-dose drug substances and facilitating the wetting and dissolution of poorly soluble, hydrophobic drug substances.

Dry granulation: Can be produced by passing powders between rollers at elevated pressure (roll compaction). Alternatively, dry granulation can also be carried out by the compaction of powders at high pressures on tablet presses, a process also known...
as slugging. In either case, the compacts are sized before compression. Dry granulation improves the flow and handling properties of the powder formulation without involving moisture in the processing.

**Direct compression**: Tablet processing involves dry blending of the drug substance(s) and excipients followed by compression. The simplest manufacturing technique, direct compression, is acceptable only when the drug substance and excipients possess acceptable flow and compression properties without prior process steps.

Tablets may be coated to protect the ingredients from air, moisture, or light; to mask unpleasant tastes and odors; to improve tablet appearance; and to reduce dustiness. In addition, coating may be used to protect the drug substance from acidic pH values associated with gastric fluids or to control the rate of drug release in the gastrointestinal tract.

The most common coating in use today is a thin film coating composed of a polymer that is derived from cellulose. Sugar coating is an alternative, less common approach. Sugar-coated tablets have considerably thicker coatings that are primarily sucrose with a number of inorganic diluents. A variety of film-coating polymers are available and enable the development of specialized release profiles. These formulations are used to protect acid-labile drug substances from the acidic stomach environment as well as to prolong the release of the drug substance to reduce dosing frequency (see (711) or (701)).

**Tapes**

A tape is a dosage form suitable for delivering drug substances to the skin. It consists of a drug substance(s) impregnated into a durable yet flexible woven fabric or extruded synthetic material that is coated with an adhesive agent. Typically the impregnated drug substance is present in the dry state. The adhesive layer is designed to hold the tape securely in place without the aid of additional bandaging. Unlike transdermal systems, tapes are not designed to control the release rate of the drug substance. The term “tape” is not preferred and should not be used for new official article titles.

The drug substance content of tapes is expressed as amount per surface area with respect to the tape surface exposed to the skin. The use of an occlusive dressing with the tape enhances the rate and extent of delivery of the drug substance to deeper layers of the skin and may result in greater systemic absorption of the drug substance.

**GLOSSARY**

This glossary provides definitions for terms in use in medicine and serves as a source of official titles for official articles, except when the definition specifically states that the term is not to be used in drug product titles. Examples of general nomenclature forms for the more frequently encountered categories of dosage forms appear in (1121). In an attempt to be comprehensive, this glossary was compiled without the limits imposed by current preferred nomenclature conventions. To clearly identify/distinguish preferred from not preferred terms, entries indicate when a term is not preferred and generally direct the user to the current preferred term. Descriptive terms are used to identify a specialized presentation or characteristic of a dosage form. For example, the descriptive term “chewable” may be used with the dosage form “tablets” to identify a specific type of tablet that must be chewed prior to swallowing.

**Aerosol**: A dosage form consisting of a liquid or solid preparation packaged under pressure and intended for administration as a fine mist. When not used in naming, the term “aerosol” also refers to the fine mist of small droplets or solid particles that are emitted from the product.

**Aromatic water** (see **Solution**): A clear, saturated, aqueous solution of volatile oils or other aromatic or volatile substances. The term is not used in official article titles.

**Aural (Auditory)** (see **Otic**): For administration into, or by way of, the ear. The term is not used in official article titles.

**Bead** (see **Pellet**): A solid dosage form in the shape of a small sphere. In most products a unit dose consists of multiple beads. The term is not used in official article titles.

**Bolus** (not preferred; see **Tablet**): A large tablet intended for administration to large animals. Occasionally, the term “bolus” is used to describe a method of administration.

**Buccal**: Administration directed toward the cheek, generally from within the mouth.

**Caplet** (see **Tablet**): Tablet dosage form in the shape of a capsule. The term is not used in official article titles.

**Capsule**: A solid dosage form in which the drug substance, with or without other ingredients, is filled into either a hard or soft shell or coated on the capsule shell. Most capsule shells are composed mainly of gelatin.

**Chewable**: A term for a solid dosage form that is intended to be chewed or crushed before swallowing.

**Chewable gel**: Formed or molded oral gel dosage forms that maintain their shape, are elastic, and yield to mastication. Chewable gels are also known as “gummies” but that term is not used for official article titles.

**Coating (Coated)**: A term for the outer solid covering applied to a solid dosage form. This outer deposit is also referred to as a film. Coatings are applied for functional or aesthetic purposes such as taste masking, stability, modifying release characteristics, product identification, and appearance. The term is not used in official article titles.

**Collodion** (not preferred; see **Solution**): A preparation that is a solution dosage form composed of pyroxilin dissolved in a solvent mixture of alcohol and ether, and applied externally.

**Colloidal dispersion**: A term for a preparation or formulation in which particles of colloidal dimension (i.e., typically between 1 nm and 1 µm) are distributed uniformly throughout a liquid.

**Concentrate** (not a preferred term for human or veterinary drug products): The current use is for drug substances that are not intended for direct administration to humans or animals. The use in drug product nomenclature is being phased out (see (1121) and **Nomenclature Guidelines**).

**Conventional-release** (see **Immediate-release**): A term describing a dosage form in which no deliberate effort has been made to modify the release rate of the drug substance. In the case of capsules and tablets, the inclusion or exclusion of a disintegrating agent is not interpreted as a modification. The term is not used in official article titles.

**Cough drop** (see **Lozenge**): The term is not used in official article titles.

**Cream**: A semisolid emulsion dosage form often containing more than 20% water and volatiles, and/or containing less than 50% hydrocarbons, waxes, or polyols as the vehicle for the drug substance. Creams are generally intended for external application to the skin or mucous membranes.
Delayed-release: A type of modified-release dosage form. When used in naming dosage forms, this term denotes a dosage form deliberately formulated to delay release of the drug substance for some period of time after initial administration. For oral products, expressions such as “enteric-coated” or “gastro-resistant” have been used where release of the drug substance is prevented in the intestinal environment but promoted in the gastric environment. However, the term “delayed-release” is used for official article titles.

Dental: When used in naming dosage forms, this term denotes a preparation that is applied to the teeth for localized action.

Dip (not preferred; see Immersion)

Dispersible tablet (see Tablet, Tablet for oral suspension, or Tablet for oral solution): The term is not used in official article titles.

Disintegrating tablet (see Tablet, Tablet for oral suspension, or Tablet for oral solution; see also Orally disintegrating): The term is not used in official article titles.

Dry powder inhaler: A device used to administer an inhalation powder in a finely divided state suitable for oral inhalation by the patient. This term is not used in official article titles.

Effervescent: A term for an oral dosage form, frequently tablets or granules, containing ingredients that, when in contact with water, rapidly release carbon dioxide. The dosage form is dissolved or dispersed in water to initiate the effervescence prior to ingestion.

Elixir (not preferred; see Solution): A preparation that typically is a clear, flavored, sweetened hydroalcoholic solution intended for oral use. The term should not be used for new drug products in USP–NF but is commonly encountered in compounding pharmacy practice.

Emollient: A term for a cream or ointment indicating an increase in the moisture content of the skin following application of bland, fatty, or oleaginous substances. This term should not be used in official article titles.

Emulsion: A dosage form consisting of a two-phase system composed of at least two immiscible liquids, one of which is dispersed as droplets (internal or dispersed phase) within the other liquid (external or continuous phase), generally stabilized with one or more emulsifying agents. Emulsion is not used as a dosage form term if a more specific term is applicable (e.g., Cream, Lotion, or Ointment).

Enteric-coated (see Delayed-release): A term for a solid dosage form in which a polymer coating has been applied to prevent the release of the drug substance in the gastric environment. This term is not used in official article titles.

Excipient: An ingredient of a dosage form other than a drug substance. This term is not used in official article titles. The term “excipient” is synonymous with inactive ingredient.

Extended-release: A term denoting a dosage form that is deliberately formulated to prolong the release of the drug substance compared to that observed for an immediate-release dosage form. Expressions such as “prolonged release”, “repeat action”, “controlled release”, “long acting”, and “sustained release” have also been used to describe such dosage forms. However, the term “extended-release” is used for official article titles.

Extended-release injectable suspension: Liquid preparations of solids suspended in a suitable vehicle and formulated to allow the drug substance to be available over an extended period of time. The term ”for extended-release injectable suspension” indicates dry solids that, upon the addition of a suitable vehicle, yield a preparation that conforms in all respects to the requirements for extended-release injectable suspensions.

Film: A term used to describe a thin sheet of material, usually composed of a polymer. Films are used in various routes of administration including as a means of oral administration of material in a rapidly dissolving form.

Foam: A dosage form containing gas dispersed in a liquid or solid continuous phase. Foams are formed at the time of application by dispensing product from the canister or other appropriate container and can be formulated to quickly break down into a liquid or to remain as a foam to ensure prolonged contact.

Gas: One of the states of matter having no definite shape or volume and occupying the entire container when confined. The term is not used in official article titles.

Gastro-resistant (see Delayed-release): A term for a solid dosage form in which a polymer coating has been applied to prevent release in the gastric environment. The term is not used in official article titles.

Gel: A dosage form that is a semisolid dispersion of small particles or a solution of large molecules interpenetrated by a solution containing a gelling agent to provide stiffness.

Gelcap: A capsule that is coated is sometimes referred to as a gelcap. Gelcap is not a term used in official article titles.

Geltab/Filmtab: A tablet that is coated is sometimes referred to as a geltab or filmtab. Geltab and filmtab are not terms used in official article titles.

Granules: A dosage form composed of dry aggregates of powder particles that may contain one or more drug substances, with or without other ingredients. They may be swallowed as such, dispersed in food, or dissolved in water. Granules are frequently compacted into tablets or filled into capsules, with or without additional ingredients. More commonly, granules are reconstituted as suspensions.

 Gum: A dosage form in which the base consists of a pliable material that, when chewed, releases the drug substance into the oral cavity.

Gummies (see Chewable gel): The term is not used in official article titles.

Hard-shell capsule (not preferred; see Capsule): A type of capsule in which one or more drug substances, with or without other ingredients, are filled into a two-piece shell. Most hard-shell capsules are composed mainly of gelatin and are fabricated prior to the filling operation.

Immediate-release: A term for a dosage form in which no deliberate effort has been made to modify the drug substance release rate. The term is not used in official article titles.

Immersion: A veterinary route of administration via partial or complete submersion in a specified environment such as liquid or air.
Implant: A dosage form that is a solid or semisolid material containing the drug substance that is placed into the body. The implantation process is invasive, and the material is intended to reside at the site for a period consistent with the design release kinetics or profile of the drug substance(s).

Inhalation (By Inhalation): A route of administration for aerosols characterized by dispersion of the drug substance into the airways during inspiration.

Injection (By Injection): A route of administration of a liquid or semisolid deposited into a body cavity, fluid, or tissue by use of a needle.

Injection: Liquid preparations that may contain drug substances and/or excipients or solutions thereof. The term “for injection” indicates dry solids that, upon the addition of a suitable vehicle, yield solutions conforming in all respects to the requirements for injections.

Injectable emulsion: Liquid preparations of drug substances dissolved or dispersed in a suitable emulsion medium.

Injectable suspension: Liquid preparations of solids suspended in a liquid medium. The term “for injectable suspension” indicates dry solids that, upon the addition of a suitable vehicle, yield preparations conforming in all respects to the requirements for injectable suspensions. For extended-release preparations, see Extended-release injectable suspension.

Insert: A solid dosage form that is inserted into a naturally occurring (nonsurgical) body cavity other than the mouth or rectum. It should be noted that a suppository is intended for application into the rectum and is not classified as an insert (see Suppository).

Intraocular: A route of administration to deliver a sterile preparation within the eye.

Irrigation: A sterile solution or liquid intended to bathe or flush open wounds or body cavities.

Jelly (not preferred; see Gel): A semisolid dispersion of small particles or a solution of large organic molecules interpenetrated by a solution containing a gelling agent to promote stiffness.

Liposomes: A term for preparations of amphiphilic lipids that have low water solubility (see (1)).

Liquid: A dosage form consisting of a pure chemical in its liquid state. This dosage form term should not be applied to solutions. The term is not used in official article titles. When not used in dosage form naming, the term, “liquid” is used to indicate a material that is pourable and conforms to its container at room temperature.

Lotion: An emulsion liquid dosage form applied to the outer surface of the body. Historically, this term was applied to topical suspensions and topical emulsions. The current definition of a lotion is restricted to an emulsion.

Lozenge: A solid dosage form intended to disintegrate or dissolve slowly in the mouth.

Modified-release: A term for a dosage form with a drug substance release pattern that has been deliberately changed from that observed for the immediate-release dosage form of the same drug substance. The two types of modified-release are extended-release and delayed-release. The term “modified-release” is not used in official article titles.

Molded tablet: A tablet that has been formed by dampening the ingredients and pressing into a mold, then removing and drying the resulting solid mass. This term is not used in official article titles.

Mouthwash (see Rinse): Term applied to a solution preparation used to rinse the oral cavity. The term is not used in official article titles.

Nasal: Route of administration (mucosal) characterized by administration to the nose or by way of the nose for local or systemic effect.

Ocular (not preferred; see Intraocular): Route of administration indicating deposition of the drug substance within the eye.

Ointment: A semisolid dosage form, usually containing less than 20% water and volatiles and more than 50% hydrocarbons, waxes, or polyols as the vehicle. This dosage form generally is for external application to the skin or mucous membranes.

Ophthalmic: A route of administration characterized by application of a sterile preparation to the external parts of the eye.

Oral: Route of administration characterized by application to the mouth or delivery to the gastrointestinal tract through the mouth.

Orally disintegrating: When used in naming a dosage form, this term denotes a solid oral dosage form that disintegrates rapidly in the mouth prior to swallowing. The drug substance is intended for gastrointestinal delivery and/or absorption. See also CDER Guidance for Industry: Orally Disintegrating Tablets.

Oral dispersible (see Orally disintegrating): The term is not used in official article titles.

Oro-pharyngeal: A route of administration characterized by deposition of a preparation into the oral cavity and/or pharyngeal region to exert a local or systemic effect.

Otic: A route of administration characterized by deposition of a preparation into, or by way of, the ear. Sometimes referred to as Aural (Aural not preferred).

Parenteral: General route of administration which is characterized by injection through the skin or other external boundary tissue or implantation within the body. Specific parenteral routes include intravenous, intraventricular, intra-arterial, intra-articular, subcutaneous, intramuscular, intrathecal, intracisternal, and intracocular (see (1)).

Paste: A semisolid dosage form containing a high percentage (20%–50%) of finely dispersed solids with a stiff consistency. This dosage form is intended for application to the skin, oral cavity, or mucous membranes.

Pastille (see Lozenge): The term is not used in official article titles.

Patch (not preferred; see System): Frequently incorrectly used to describe a System.

Pellet: A small solid dosage form of uniform, sometimes spherical, shape intended for direct administration. Spherical pellets are sometimes referred to as Beads. Pellets used in veterinary medicine are typically cylindrical in shape. Pellets intended as implants must be sterile, except for some ear implants used in animal drugs. The use of the term “pellet” for implantable dosage forms is no longer preferred (see Implant).

Periodontal: A term for a preparation that is applied around a tooth for localized action.

Pill: A solid, spherical dosage form usually prepared by a wet massing, piping, and molding technique. This term is frequently incorrectly used as a general term to describe solid oral dosage forms such as tablets or capsules.

Plaster (not preferred): A dosage form containing a semisolid composition supplied on a support material for external application. Plasters are applied for prolonged periods of time to provide protection, support, or occlusion (for macerating action).
**Powder**: A dosage form composed of a solid or mixture of solids reduced to a finely divided state and intended for internal or external use.

**Powder, inhalation**: A powder containing a drug substance for oral inhalation. The powder is used with a device that aerosolizes and delivers an accurately metered amount.

**Premix** (not preferred; see Animal Drugs for Use in Animal Feeds (1152), Scope, Type A Medicated Articles and Type B Medicated Feeds)

**Prolonged-release** (see Extended-release): The term is not used in official article titles.

**Rectal**
- **Solution**: A clear, homogeneous liquid dosage form that contains one or more chemical substances dissolved in a solvent or mixture of mutually miscible solvents.
- **Suppository**: A solid dosage form intended to disintegrate or dissolve slowly in the rectum and usually prepared for oral or external use.

**Soap**: The alkali salt(s) of a fatty acid or mixture of fatty acids used to cleanse the skin. Soaps used as dosage forms may contain a drug substance intended for topical application to the skin. Soaps have also been used as liminents and enemas.

**Soft gel capsule** (not preferred; see Capsule): A specific capsule type characterized by increased levels of plasticizers producing a more pliable and thicker-walled material than hard gelatin capsules. Soft gel capsules are further distinguished because they are single-piece sealed dosages. Frequently used for delivering liquid compositions.

**Solution**: A clear, homogeneous liquid dosage form that contains one or more chemical substances dissolved in a solvent or mixture of mutually miscible solvents.

**Spirit** (not preferred; see Solution): A liquid dosage form composed of an alcoholic or hydroalcoholic solution of volatile substances.

**Spot on (Pour On)**: A method of delivering liquid veterinary drug products by administering them onto the animal’s skin, usually between the shoulder blades (spot on) or down the back (pour on). The term is not used in official article titles.

**Spray**: A spray is a dosage form that contains drug substance(s) in the liquid state, either as a solution or as a suspension, and is intended for administration as a mist. Sprays are distinguished from aerosols in that spray containers are not pressurized. Most of the sprays are generated by manually squeezing a flexible container or actuation of a pump that generates the mist by discharging the contents through a nozzle.

When not used in the naming of a dosage form, the term “spray” describes the generation of droplets of a liquid or solution to facilitate application to the intended area.

**Stent, drug-eluting**: A specialized form of implant used for extended local delivery of the drug substance to the immediate location of stent placement.

**Strip** (only used for diagnostic products, otherwise not preferred; see Film): A dosage form or device in the shape of a long, narrow, thin, absorbent, solid material such as filter paper.

**Sublingual**: A route of administration characterized by placement underneath the tongue and for release of the drug substance for absorption in that region.

**Suppository**: A solid dosage form in which one or more drug substances are dispersed in a suitable base and molded or otherwise formed into a suitable shape for insertion into the rectum to provide local or systemic effect.

**Suspension**: A liquid dosage form that consists of solid particles dispersed throughout a liquid phase.

**Syrup** (not preferred; see Solution): A solution containing high concentrations of sucrose or other sugars. This term is commonly used in compounding pharmacy.

**System**: A preparation of drug substance(s) in a carrier device that is applied topically or inserted into a body cavity. The drug substance is designed to be released in a controlled manner over a specified period of time or the drug substance is released based on its concentration in the formulation. Unless otherwise stated in the labeling, the carrier device is removed after use.

**Tablet**: A solid dosage form prepared from powders or granules by compaction.

**Tablet for oral solution**: A tablet that is intended to be dispersed in a liquid before administration. When dispersed in the liquid, a solution results.

**Tablet for oral suspension**: A tablet that is intended to be dispersed in a liquid before administration. When dispersed in the liquid, a suspension results.

**Tape** (not preferred): A dosage form or device composed of a woven fabric or synthetic material onto which a drug substance is placed, usually with an adhesive on one or both sides to facilitate topical application. The rate of release of the drug substance is not controlled.

**Tincture** (not preferred; see Solution): An alcoholic or hydroalcoholic solution prepared from vegetable materials or from chemical substances.

**Transdermal**: A route of administration characterized by drug product application to the skin where the drug substance passes through the dermal layer with the intent to achieve a systemic effect.

**Troche** (see Lozenge): A solid dosage form intended to disintegrate or dissolve slowly in the mouth and usually prepared by compaction in a manner similar to that used for tablets. The term is not used in official article titles.

**Urethral**: A route of administration characterized by deposition into the urethra.

**Vaginal**: A route of administration characterized by deposition into the vagina.

**Vehicle**: A term commonly encountered in compounding pharmacy that refers to a component for internal or external use that is used as a carrier or diluent in which liquids, semisols, or solids are dissolved or suspended. Examples include water, syrups, elixirs, oleaginous liquids, solid and semisolid carriers, and proprietary products (see Excipient). This term is not used in official article titles.

**Veterinary**: A term for dosage forms intended for nonhuman use.
Change to read:

STERILITY ASSURANCE

INTRODUCTION

This informational chapter provides general information on the concepts and principles involved in the preparation of materials that must be sterile. Within the strictest definition of sterility, an item is deemed sterile only when it contains no viable microorganisms. However, this textual definition cannot be applied to actual items labeled as sterile because of irresolvable limitations in testing. Sterility cannot be demonstrated without the destructive testing of every sterile unit. In a real sense, microbiological safety is achieved through the implementation of interrelated controls that in combination provide confidence that the items are suitable for use as labeled. It is the controls that provide the desired assurance from microbiological risk rather than the results of any in-process or finished goods testing. The verification of safety of products labeled sterile is generally known as “sterility assurance” and that nomenclature will be used throughout this chapter.

The establishment of an effective sterility assurance program requires information about the material to be sterilized. An initial determination should be made regarding the potential for terminal sterilization of the material in its primary container applying the principles defined in Sterilization of Compendial Articles. As described, the appropriate process provides a balance between conditions that are lethal to potential bioburden present in/on the item and those that preserve its essential quality attributes. Depending upon the results of that determination, sterility of the item may be achieved by either aseptic processing or terminal sterilization. The potential for a process that relies on both the protective measures inherent to aseptic processing and the lethal nature of terminal sterilization may offer advantages. Regardless of the process selection decision, the establishment of design, operation, process controls, and monitoring systems is essential to provide the necessary confidence in the outcome.

The production of sterile products is subject to numerous factors that influence the outcome (see Figure 1). The identified factors in the image should be considered for their impact on sterility of the final product, although not all of the influencing elements are depicted.

The decisions made relative to the influences shown in Figure 1 will determine the success of the sterility assurance program. Poor choices, regardless of any successful process controls associated with them, must be acknowledged as fundamentally unsound. The process design objective is related to contamination controls intended to obviate risk of microbial ingress. This focus is appropriate regardless of whether the process used is aseptic processing or terminal sterilization.

Recognition that operating personnel are the most significant contributors of microbiological risk leads to design preferences and operating principles that should be adhered to with respect to sterile operations. This knowledge underscores the importance of separating personnel from the aseptic environment and limiting their interaction with sterilized components and product(s). The means for accomplishing these goals are embodied in two complementary practices:

- The use of automation technology—to reduce or eliminate personnel interventions and thus personnel-borne contamination
- The use of separative technologies—to eliminate, to the extent technically possible, human sourced contamination

Thus the implementation of appropriate contamination control procedures is paramount in design and operation of sterile product manufacturing systems.

Consideration of these principles adapts the Quality by Design (QbD) approach widely adopted in regulatory standards. Using QbD concepts in sterile operations is markedly different from the applications in the typical formulation, pharmaceutical, chemical, or biological synthesis process. The establishment of direct linkage between a monitored condition and process outcome with respect to sterile manufacturing is statistically difficult and analytically uncertain. The situation with...
respect to the definition of physical design elements is similar. Given the great variation in sterile product manufacturing with respect to scale, configuration, and complexity, it follows that the design alternatives and operating practices must also be flexible. Thus, the recommendations provided in this chapter are entirely non-numeric, because there are no ready means with which to demonstrate the suitability of specific values. Instead, QbD for sterile processing should be driven toward a singular goal of optimizing contamination control with a particular focus on the microbial risk impact of personnel. The specific means vary but should be of prime consideration in process design. Figure 2 outlines the elements that contribute to sterility assurance, as described in the remaining sections of this chapter.

**Figure 2. Elements contributing to sterility assurance.**

### Aseptic Processing

There are a substantial number of sterile products that cannot be terminally sterilized because of adverse impact on the product/package’s essential properties and must be prepared by aseptic processing. Aseptic processes are designed to prevent the introduction of viable microorganisms into/onto separately sterilized materials during their assembly into a sealed sterile package. Aseptic processes can vary in complexity from comparatively simple filling/sealing to challenging and lengthy manufacturing sequences required for complex items. Regardless of process scale, all of the individually sterilized materials must be protected from contamination from the point of sterilization through closure of the primary package. This is accomplished through adherence to the principles described below in which an International Organization for Standardization (ISO) 5 condition is maintained when materials are exposed to the environment (see Microbiological Control and Monitoring of Aseptic Processing Environments (1116)). Exclusive to aseptic processing is the execution of process simulations that support batch or campaign duration (4–6).

### Terminal Sterilization

Terminally sterilized products are the lowest risk category of sterile pharmaceutical products. Unlike products aseptically manufactured under conditions designed to prevent microbial ingress, terminally sterilized products are subjected to a sterilization process that imparts a quantifiable safety level. Terminal sterilization processes achieve this by delivering measurable physical conditions that correspond to microbial lethality. For terminally sterilized products, sterility assurance is defined in terms of the probability of nonsterility (PNS), or the probability of the terminal sterilization process generating a nonsterile unit (PNSU). Terminal sterilization processes must achieve a consistent validated performance of a PNSU of \( \leq 10^{-6} \) (a probability of NMT 1 nonsterile unit in 1 million units produced) (see Chapter (1229)). The convention by which terminal sterilization cycles are developed and validated ensures that the actual PNSU is typically much lower (better) than the minimum standard of \( <10^{-6} \). Chapter (1229) summarizes the common requirements for sterilization process design, development, validation, and process control. Terminal sterilization processes share common requirements of well-defined process parameters strictly controlled within defined operating limits. Terminal sterilization must be supported by a system of product disposition, which includes the assessment of critical physical process parameters, presterilization product parameters (e.g., bioburden, container–closure integrity), and environmental parameters. Terminal sterilization can rely on parametric release practices to obviate the need for sterility testing (see Terminally Sterilized Pharmaceutical Products—Parametric Release (1222)).

### Post-Aseptic Processing Terminal Sterilization

An aseptic process followed by a terminal sterilization process provides superior control over the presterilization bioburden, such that the subsequent sterilization process can be designed with less overall lethality, thereby making it possible to substantially extend the use of terminal sterilization to products with greater sensitivity to the applied energy of the process. From a patient safety perspective, this approach has the following distinct advantages:
An adventitious contaminant introduced during aseptic processing is easily killed by the terminal sterilization step, reducing the extent of in-process environmental monitoring performed.

Bioburden controls for the terminal process are simplified because all units have been aseptically filled.

Where a product is made using either process alone, the limitations of each (no terminal lethal component in aseptic, more degradation in terminal sterilization) would persist.

Where bioburden is controlled through aseptic processing, terminal sterilization can be applicable at lower lethality levels. Classical Fα, time-temperature, and radiation dose (kGy) targets for sterilization processes are arbitrarily selected and intended to simplify process validation, but in reality serve to reduce the use of terminal treatments. Physical lethality data based on fixed numerical values are inherently conservative and disregard the degradative impact of the sterilization process on the product. The focus must be on the ability of the process to kill bioburden organisms rather than biological indicators (see 1229 and Moist Heat Sterilization of Aqueous Liquids (1229.2)).

While product quality attributes can be impaired by “standard” sterilizing conditions, the combined process can utilize less aggressive sterilizing conditions to minimize adverse effects upon the product and primary packaging materials. If the terminal treatment follows aseptic processing, then the sterilizing conditions need not be excessive as there is essentially no risk from presterilization bioburden in the filled containers. Sterilization process conditions would be dictated by the specifics of the product in parallel with the establishment of appropriate controls on presterilization bioburden derived from environmental and prefiltration isolates.

Containers and Closures

The container and closure for a sterile formulation are integral parts of the sterile product. The container materials provide essential protection to the product throughout its shelf life and are chosen to minimize interaction with, preserve the quality attributes of, and facilitate dispensing of the sterile product. The container materials should be readily sterilizable, either separately prior to filling and/or together with the formulation in a terminal process. Containers and closures should be selected for:

- Reliability of container–closure integrity over the shelf life
- Absence of interaction with formulation materials
- Ease of handling in the processing environment and during administration
- Tolerance of variation in equipment and other components
- Cleanliness including freedom from particulates and absence of leachable or extractable chemicals
- Compatibility with the product
- Control over endotoxin content (where appropriate)
- Protection of components prior to use (where appropriate)
- Compatibility with preparation, sterilization, and depyrogenation processes (where appropriate)

The essential aspects of container–closure materials for sterile products are subject to numerous requirements elsewhere within USP–NF. The reader must consider the content provided in Injections and Implanted Drug Products (1), Bacterial Endotoxins Test (85), Visible Particulates in Injections (790), Package Integrity Evaluation—Sterile Products (1207), Depyrogenation (1228), and (1229).

Decontamination

Decontamination is a broadly defined term used to describe a variety of processes that reduce microbial populations without an expectation for total kill. It is not a substitute for sterilization; a sterilization process should be used wherever possible. A variety of chemical agents and methods are used that vary depending upon the application. Decontamination is used for bioburden reduction of materials, equipment, and environments in support of sterile product manufacture:

- For materials and surfaces that cannot be sterilized
- For materials and surfaces that do not require sterilization

Decontamination processes are ordinarily separated into two major categories based upon their effectiveness against spore-forming microorganisms (see Disinfectants and Antiseptics (1072)). Sporicidal treatments are used in critical applications such as isolator decontamination, air-lock/pass-throughs, etc. Their toxicity to personnel and sometimes corrosive chemistry may preclude their exclusive use for microbial control. Non-sporicidal agents have fewer safety and material impact concerns, and the occasional use of a sporicidal agent is required to control spore populations. Applications for decontamination are diverse; among the more common uses are:

Decontamination of controlled environments and non-product contact surfaces

- In conventional cleanrooms, including restricted access barrier systems (RABS), this is predominantly a manual process performed after cleaning of the room/production line
- Decontamination of items upon transition into an environment of higher classification
- Isolators commonly use an automated process
- Periodic decontamination of operator gloves during processing

Decontamination of product contact surfaces

1 Sterilization is preferred over decontamination and should be utilized wherever possible, consistent with minimization of handling post-sterilization.
Large equipment (e.g., stopper bowls) can be manually sanitized on a frequent basis in addition to sterilization to avoid the extensive manipulation required for their installation post-sterilization\(^2\). Re-decontamination of sterilized equipment after aseptic assembly or intervention. Periodic decontamination of previously sterilized utensils prior to interventions.

**Depyrogenation**

The minimization of pyrogen content is a requirement for injectable products. During the production of sterile products, depyrogenation processes are used in a variety of ways to minimize pyrogenic contamination of surfaces, materials, and products. Details on depyrogenation processes are provided in (1228).

**Equipment**

Equipment used for sterile product manufacturing varies in its impact on the manufacturing process and on product quality and should have several important characteristics. For example, the equipment should:

- Operate reliably and produce products of consistent quality
- Not adversely impact essential product quality attributes
- Be easily cleanable and sterilizable, as necessary
- Minimize human intervention during set-up and operation through such features as physical separation, automation, and robotics
- Be tolerant of variations in container–closure materials
- Designed to minimize product exposure to the background environment

The extent to which the equipment interacts with process materials and the product affects the level of impact. Process equipment can influence the quality of the finished product in a variety of ways, and this can occur prior to and after sterilization and depyrogenation.

**EQUIPMENT IN DIRECT CONTACT WITH COMPONENTS, CONTAINERS, CLOSURES, AND STERILE PRODUCTS**

This equipment category includes those items in direct contact with the drug substance, drug product, raw materials, and primary packaging components, including, for example, mixing and storage vessels, piping systems and tubing, filters, filling pumps, lyophilizer shelves, and feed hoppers. Product contact surfaces of this equipment are designed and may require additional treatment to minimize adverse impact (microbial, particulate, and chemical) on the contacted materials. The procedures used for the cleaning and sterilization of direct contact surfaces, including dirty, clean, and sterile hold times, must be validated to ensure they do not adversely impact essential product quality attributes as well as to verify the effectiveness of the cleaning procedure and that no microbial recontamination/proliferation occurs during equipment storage. Direct contact utensils are subject to the same considerations. With appropriate consideration of materials' compatibility, single-use disposable equipment (supplied sterile when necessary) may be utilized.

**EQUIPMENT HAVING INDIRECT CONTACT WITH COMPONENTS, CONTAINERS, CLOSURES, AND STERILE PRODUCTS**

Equipment having a significant impact on product quality, that does not contact components, primary packaging materials, and sterile products, includes the electro-mechanical elements (non-product contact) of filling machines, stoppering machines, and sterilizers. The performance of this equipment can change fill weight, particle size, moisture level, content uniformity, container–closure integrity, and other essential quality attributes. This equipment is ordinarily located near exposed product contact equipment surfaces. For example, a pre-assembled filling set (product contact equipment) may be installed on a filling machine (significant impact without product contact), which provides control over the fill volume or weight. The surfaces of this equipment must be compatible with the cleaning and microbial decontamination and/or sterilization agents employed.

**OTHER EQUIPMENT**

Some equipment has only an indirect impact on product quality, for example, conveyors, turntables, balances, air samplers, and carts. The influence of this equipment is largely on the environments in which the product is made. The exposed surfaces of this equipment must be compatible with the cleaning and decontamination agents used.

**Facilities**

Sterile manufacturing operations are supported by administrative, laboratory, maintenance, and warehouse functions and other activities. The impact of these operations on the location and overall design of the sterile manufacturing area must be considered. Emphasis should be given in facility design to the flows of materials, components, personnel, equipment, and waste streams throughout the facility and to the orderly transition of items between environments of different classifications to prevent mix-up and avoid product contamination. Facility environmental and utility systems must be designed to minimize microbial, chemical, and particulate contamination. The facility design must be supported by practices and procedures such as cleaning and decontamination, gowning, and material transfer. The architectural details of the facility infrastructure must consider the

\(^2\) Sterilization out-of-place would be used on a less frequent basis.
means for cleaning and disinfection. Detailed design recommendations can be found in the International Society for Pharmaceutical Engineering’s Baseline Guide: Sterile Product Manufacturing Facilities (7).

The core activities for sterile product manufacture are carried out in classified environments operating in conformance with the ISO 14644 series of standards (8). A pressure cascade descending from the more critical areas to less critical is commonplace. In general, the more protection that materials have from potential sources of contamination during holding or processing, the less impact the facility has on the process outcome. Human operators within ISO 14644 classified cleanrooms used in aseptic processing are the greatest risk to product safety; therefore, no single risk mitigation factor in aseptic processing is more important than minimizing risk emanating from gowned operators.

Early-stage container–closure and equipment washing and preparation are carried out in lower classification areas (ISO 7–8). Nonsterile formulation is typically carried out in ISO 6–7 environments. The production materials are introduced into the processing area where subsequent steps are performed. The bioburden level of the materials influences the detailed design of the facility and its controls. Nonsterile materials (e.g., formulation, containers, closures, equipment, and utensils) require subsequent sterilization and, where necessary, depyrogenation. Sterile materials are introduced through airlocks and pass-throughs. The facility design controls for the background, and processing environments should be chosen to preserve the intended microbial attributes of the in-process and finished materials.

Table 1 provides some examples of formulation and filling environments.

Table 1. Examples of Environments for Processing

<table>
<thead>
<tr>
<th>Processing Technology</th>
<th>Background Environment</th>
<th>Processing Environment</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conventional cleanroom</td>
<td>ISO 5–7</td>
<td>ISO 5</td>
<td>FDA AP 2004 (9), EMA Annex (10)</td>
</tr>
<tr>
<td>Closed RABS</td>
<td>ISO 7</td>
<td>ISO 5</td>
<td>ISPE (11), PHSS (12)</td>
</tr>
<tr>
<td>Open RABS</td>
<td>ISO 5</td>
<td>ISO 5</td>
<td>ISPE (11), PHSS (12)</td>
</tr>
<tr>
<td>Closed isolators</td>
<td>CNC</td>
<td>ISO 5</td>
<td>FDA AP 2004 (9), PDA TR No: 34 (13), ISPE (7)</td>
</tr>
<tr>
<td>Open isolators</td>
<td>CNC-ISO 8</td>
<td>ISO 5</td>
<td>FDA AP 2004 (9), PIC/S (14), PDA TR No: 34 (13), ISPE (7)</td>
</tr>
<tr>
<td>Blow-fill-seal/form-fill-seal</td>
<td>ISO 5–7</td>
<td>ISO 5</td>
<td>FDA AP 2004 (9), Baseman (15)</td>
</tr>
<tr>
<td>Closed systems</td>
<td>CNC</td>
<td>Not applicable(^a)</td>
<td>PDA TR No: 28 (5); Agalloco, Hussong, et al. (16)</td>
</tr>
<tr>
<td>Terminal sterilization</td>
<td>ISO 8</td>
<td>ISO 5–7</td>
<td>EMA Annex (10)</td>
</tr>
</tbody>
</table>

\(^a\) Controlled non-classified—a non-classified controlled environment with filtered air supply.
\(^b\) As the process occurs with a closed system, there is no separate processing environment.

[NOTE—Table values represent the operational condition and are adapted from the reference documents.]

**CONVENTIONAL CLEANROOM**

The critical activities are performed in ISO 5 environments supported by surrounding ISO 5–7 environments where gowned personnel are normally located. There may be only limited separation between gowned personnel and sterile materials and product contact surfaces. The critical activities are performed within a unidirectional airflow environment. Decontamination of the cleanroom is commonly performed by personnel.

**RESTRICTED ACCESS BARRIER SYSTEMS**

The typical RABS provides ISO 5 unidirectional air within the barrier and is situated in a conventional ISO 5–7 cleanroom. RABS may be designed to allow for opening of barriers to enable human intervention, or they may be designed to operate closed with the same operational restrictions regarding operator access that applies to isolators (see below). Air overspill from within the barrier is designed to prevent the ingress of contamination. Operator manipulation of sterile items is achieved using glove ports, and material transfers are accomplished without opening the system. A RABS that is opened mid-process should be treated as a conventional cleanroom (see above). RABSs require decontamination prior to use. This may be accomplished either manually or using automated systems.

**ISOLATORS**

Isolators provide complete separation between personnel and the enclosed ISO 5 processing environment. A defined pressure differential is maintained between the ISO 5 environment and the surrounding area. Air overspill provides an aerodynamic seal at points where the product exits the isolator into an external environment of lesser classification or no classification. The use of unidirectional air is not required in isolators. Isolators are commonly decontaminated using automated systems.

**BLOW-FILL-SEAL AND FORM-FILL-SEAL**

These technologies form, fill, and seal flexible walled containers in an ISO 5 environment. Blow-fill-seal (BFS) and form-fill-seal (FFS) equipment configurations allow for installation in a variety of background environments. The critical activities are
performed within a unidirectional airflow environment. Decontamination is performed as is common for the background environment.

CLOSED SYSTEMS

These systems provide for complete separation of production materials from personnel and surrounding environment. Closed systems can be single- or multiple-use vessels/chambers with means for materials ingress/egress. The designs avoid any human interaction or environmental contact with sterile materials. These systems vary in complexity and are sterilized either in situ or prior to use.

TERMINAL STERILIZATION

Filling systems and environments for containers to be terminally sterilized can be accomplished in ISO 7 or better environments. The critical activities are performed within a unidirectional airflow environment. Decontamination of the cleanroom is commonly performed by personnel.

Materials (Active Pharmaceutical Ingredients, Excipients, and Process Aids)

The preparation of sterile products encompass a wide range of materials including active pharmaceutical ingredients (small and large molecules), excipients, solvents (usually water), process gases and processing aids, all of which contribute to the microbiological quality attributes of the product. Depending upon the product being manufactured, this can require consideration of bacterial, endotoxin, and particulate contamination. Specific microbiological quality testing requirements for inactive and active ingredients testing is often specified in a relevant USP–NF monograph. Requirements for microbiological testing for total aerobic bacteria, yeast and mold counts, and specified organisms are given in Microbial Enumeration Tests (61) and Tests For Specified Microorganisms (62), and the recommended but non-mandatory enumeration targets for microbiological testing are given in Microbiological Examination of Nonsterile Products: Acceptance Criteria for Pharmaceutical Preparations and Substances for Pharmaceutical Use (1111). Some specific products may require other testing for specified organisms, including viruses, as a requirement of their regulatory approval. Requirements for bacterial endotoxin testing in injectable products are covered in (85), which describes qualification of Limulus amebocyte lysate testing for acceptable levels of bacterial endotoxin. Raw material specifications must be appropriate to ensure that the manufacturing process consistently results, as demonstrated through process validation, in products conforming to the microbiological critical quality attributes.

Microbial contamination may be present on/in active pharmaceutical ingredients, excipients, and primary packaging materials. Controlling bioburden in materials and formulated product is a critical aspect of sterility assurance. The nature of the active pharmaceutical ingredient, the excipients, and the compounded product intended to be sterile are critical elements of the product knowledge. Recognizing the potential impact on critical quality attributes, the microbiological attributes of materials should be controlled through adequate supplier controls, shipping, receipt, sampling, handling, and storage. These controls should be commensurate with the microbiological risks to process controls and sterile product safety. Laboratory results should not be utilized to rationalize inadequate process controls on the part of the supplier.

The potential impact of the materials on the microbiological critical quality attributes of presterilized and finished product must be assessed by evaluating them with appropriate compendial assays or validated alternatives. Risk assessment should consider the origin of the material (e.g., fermentation, chemical synthesis, biologically derived, enzymatic, semi-synthetic synthesis, natural origin). Materials of biological origin may have higher inherent microbiological risk than materials derived solely from chemical synthesis. Materials may have inherent physicochemical properties that mitigate microbiological risks (e.g., low water activity, extreme pH, inherent antimicrobial properties) or increase microbiological risks (e.g., aqueous solution, growth supportive nutrients); this risk of supporting microbial proliferation must be assessed.

Monitoring

Environmental monitoring is employed to qualitatively assess the effectiveness of the design and operational controls to provide suitable facility hygiene. It is neither a substitute for good facility, equipment, and process design, nor compensation for deficient practices and behavior. There are inherent limitations with all forms of viable and non-viable monitoring in terms of sample size, sample location, and recovery capability that preclude their use as anything more than an indication that a facility is operating within an acceptable state of control. Monitoring provides only a snapshot in time of the actual environmental conditions and excessive sampling due to its potentially intrusive nature can actually impair product safety or generate counts unrelated to process performance by increasing activity proximate to the critical zone.

Independent of air, surface, and personnel monitoring, media fills and sterility testing provide additional (albeit analytically and statistically limited) means to evaluate the robustness of the cleanroom design, performance, and effectiveness of cleaning/decontamination procedures, personnel gowning integrity, and aseptic practices.

Environmental control can be measured only by the monitoring performed. Satisfactory monitoring performance is the result of proper design and operation as described in this chapter and not a means to establish that condition. Performance criteria are established according to the classification of the room and its usage (see (1116)).

VIABLE MONITORING

Viable monitoring consists of detecting and estimating the level of culturable microorganisms in the air, on surfaces, and on personnel. Sampling locations are defined following a risk assessment and sampling is executed by trained operators using a variety of methods including:
**Active air sampling**
**Passive air sampling**
**Viable particle counting using fluorescence technology**
**Contact-plate sampling of surfaces, gloves, and gowns**
**Swabbing of surfaces**
**Personnel monitoring**

**NON-VIABLE MONITORING**

Non-viable monitoring measures the number and size of particulates present in the air. It can be used to initially classify the cleanroom in accordance with ISO 14644-1 and to assess routine manufacturing conditions (8). When used for the purposes of monitoring, it can be performed under static conditions (no activity) and/or dynamic conditions (routine operation). Non-viable particle monitoring is performed using calibrated particle counters.

**MEDIA FILLS/ASEPTIC PROCESS SIMULATIONS**

Process simulations are exercises in which the performance of an aseptic activity is evaluated using a sterile growth medium. The medium can be directly substituted for the product or added to it. Aseptic process simulations are typically performed before the introduction of new or revised process components (e.g., products, facilities, equipment, personnel, containers and closures, and processes) and periodically thereafter (17). Process simulations should be fully representative of processing conditions and activities utilized during routine production.

**STERILITY TESTING**

The sterility test is a harmonized compendial test. It must be understood that while execution of the test is required for the release of sterile products where parametric release has not been approved, it cannot prove the sterility of the materials tested. It should be recognized that parametric release is the default mode of sterile product release.

**Personnel**

Personnel play an essential role in the preparation of sterile products. The essential activities they perform include cleaning, assembly, equipment operation, material transfer, environmental monitoring, and decontamination. While personnel are often necessary for the performance of these activities, the contamination derived from them must be prevented from entering the production materials before and after sterilization. The importance of the controls necessary to minimize exposure to and the release of human microbial contaminants in a sterile product manufacturing environment cannot be overstated.

The personnel involved in the preparation of sterile products must:

- Understand the principles of microbiology, sterilization/depyrogenation, aseptic processing, and contamination control
- Be proficient and diligent in gowning practices. Personnel required to wear aseptic gowning should periodically demonstrate their ability to properly gown
- Adhere to proper aseptic technique during all aseptic activities even when these are performed in a RABS or isolator. Periodic demonstration of these skills can be beneficial
- Be familiar with and adhere to standard operating procedures
- Practice good personal hygiene to minimize contamination potential
- Be trained in the proper and safe operation of necessary equipment
- Be monitored microbiologically after performing aseptic operations

**Procedures**

Written procedures define the operations that have been determined through validation studies and experience to be effective in controlling and facilitating the manufacture and quality of pharmaceuticals and biopharmaceuticals. Procedures are especially important for the critical processes designed to assure the sterility of terminally sterilized and aseptically produced drug products. Procedures should be periodically reviewed and evaluated to ensure they are effective and current.

**INTERVENTIONS AND INTERVENTION PROCEDURES**

There are two types of interventions associated with the aseptic production of sterile drug products. Inherent interventions are those activities that are an integral part of the aseptic process and are performed during the production of every batch. They include set-up, replenishment of components, weight and volume checks and adjustments, and environmental monitoring. Corrective interventions are those activities that correct problems and might not be performed during the production of every batch. They can be minimized and should be avoided through careful process design. Examples include stopper jams, broken and fallen glass, defective container seals, liquid leaks, and mechanical failures requiring manual correction.

Each intervention, whether inherent or corrective, should be covered by written procedures sufficiently detailed to enable personnel to perform the intervention correctly, and to perform the intervention the same way each time regardless of whomever performs it. For example, procedures should specify the number of units, their locations, and how the units are to be removed, and personnel must be trained so they can correctly execute the procedures. No intervention should be permitted...
Interventions performed during all forms of processing must be recognized as increasing the risk of contamination dissemination and are to be avoided or designed out of the process to the extent possible.

1. Interventions performed during all forms of processing must be recognized as increasing the risk of contamination dissemination and are to be avoided or designed out of the process to the extent possible.

2. Procedures for interventions should be critically reviewed to eliminate and/or simplify aseptic processes by reducing the frequency of inherent interventions and making all interventions easier to perform.

3. Interventions should be designed for minimal risk of contaminating sterile and nonsterile materials.

4. All interventions should be performed using sterilized tools whenever possible.

5. Intervention procedures should be established in detail for all inherent interventions, and more broadly for corrective interventions (where some flexibility is necessary due to greater diversity).

6. Interventions should be incorporated in periodic media fills to evaluate the aseptic practices of the operators.

Operators should initially, and periodically thereafter, be trained in all of the procedures they are expected to perform. Considerations for operator activity during the non-aseptic filling of containers should parallel those described above to minimize the potential for contamination ingress with somewhat less rigor than those needed for aseptic operations.

**Sterilization**

The most effective means for the control of microbial population is sterilization, a process that either kills or removes viable microorganisms. In the production of sterile products, sterilization processes are used to prevent microbial contamination. Terminal sterilization processes that reproducibly destroy microorganisms in the final product container are the preferred means for the production of sterile products. Sterile products that cannot be terminally sterilized rely on individual sterilization processes (e.g., steam, radiation gas, filtration) for the various materials that comprise an aseptically processed sterile product. In addition, sterilization processes are used for product contact and other non-product contact items used in a variety of applications during the preparation of sterile products to provide absolute control of bioburden. Details on sterilization processes are provided in (1229).

**Utilities**

The manufacture of sterile products requires utilities that can have a substantial impact on the final product. Some of the utilities in the facility can become an integral part of the formulated product (e.g., Water for Injection, Nitrogen) and appropriate design of the production and distribution system for these is essential. The systems for these are tightly controlled and frequent monitoring of the utilities produced is customary. These utilities may be also used in the process, and not become a part of the sterile product. Other utilities (e.g., clean steam, compressed air, Purified Water) that are used in the cleaning/decontamination of facilities, and/or preparation of equipment, containers, and closures can also be subjected to microbial control.

Utilities included in the product, in direct product contact, and in the preparation of equipment, containers, closures, and other items must meet the requirements defined in the appropriate USP–NF monograph. The systems for their preparation should be subject to formalized controls that maintain a controlled state over time. This is accomplished through a number of related practices essential for continued use of the system over an extended time. The essential practices to maintain controlled status of the utility systems include: calibration, change control, corrective and preventive maintenance, and ongoing process control.

There are other less impactful utilities (e.g., vacuum, cooling water) necessary for the operation of the facility and equipment. Although these non-product contact utilities may lack monograph requirements, their reliable operation is necessary for consistent production of sterile products.

**SUMMARY**

The safety of products labeled sterile requires that their critical quality attributes consistently meet specifications. Sterility is the most essential quality attribute. Sterility is an unqualified concept in which an item is devoid of living microorganisms capable of reproduction. Monitoring of all types, environmental (viable and non-viable; air, surface, and personnel), media fills, and sterility tests are forms of microbiological analysis that have been historically employed as proof of “sterility”. These assessment tools cannot provide definitive evidence of either “sterility” or “nonsterility”, because the means to confirm either of those conditions non-destructively is not scientifically possible. Confidence in sterile product manufacturing is realizable only by a holistic approach in which all of the supportive elements of the operations are given due consideration and emphasis (17).

The absolute nature of sterility presents the practitioner with an inherent paradox—there is no ready means to demonstrate sterility of an item in the absolute sense regardless of the means used to provide it. Test methods including those defined in this compendium (Sterility Tests (71)) utilize a number of samples taken from a large population to infer the “sterility” of the whole. Sterilization procedures including those validated for parametric release can deliver a low probability of a nonsterile unit, but not absolute assurance that “sterility” actually exists.

The uncertainty associated with proof of “sterility” notwithstanding, the means by which sterility assurance is provided are reasonably well defined. The “sterility” of any item is definitively established by the process controls summarized in this chapter rather than any form of monitoring or sampling. For terminally sterilized products, greater weight can be placed on the sterilization process utilized than on any form of testing. Confidence in aseptic processing is a result of sound design, reliable equipment, quality materials, effective procedures (including supportive sterilization processes), and personnel proficiency rather than through sampling dependent sterility testing, microbiological monitoring, and process simulation.
REFERENCES

11. International Society for Pharmaceutical Engineering. Restricted access barrier systems (RABS) for aseptic processing, ISPE definition; 2005.
14. Pharmaceutical Inspection Convention and Pharmaceutical Inspection Co-operation Scheme. PIC/S Isolators used for aseptic processing and sterility testing (PI 014-2); 2004.

1225) VALIDATION OF COMPENDIAL PROCEDURES

Test procedures for assessment of the quality levels of pharmaceutical articles are subject to various requirements. According to Section 501 of the Federal Food, Drug, and Cosmetic Act, assays and specifications in monographs of the *USP–NF* constitute legal standards. The Current Good Manufacturing Practice regulations [21 CFR 211.194(a)] require that test methods, which are used for assessing compliance of pharmaceutical articles with established specifications, must meet proper standards of accuracy and reliability. Also, according to these regulations [21 CFR 211.194(a)(2)], users of analytical methods described in *USP–NF* are not required to validate the accuracy and reliability of these methods, but merely verify their suitability under actual conditions of use. Recognizing the legal status of *USP* and *NF* standards, it is essential, therefore, that proposals for adoption of new or revised compendial analytical procedures be supported by sufficient laboratory data to document their validity. The text of this information chapter harmonizes, to the extent possible, with the International Council for Harmonisation (ICH) tripartite guideline *Validation of Analytical Procedures* and the *Methodology* extension text, which are concerned with analytical procedures included as part of registration applications submitted within the EC, Japan, and the USA.

SUBMISSIONS TO THE COMPENDIA

Submissions to the compendia for new or revised analytical procedures should contain sufficient information to enable members of the USP Council of Experts and its Expert Committees to evaluate the relative merit of proposed procedures. In most cases, evaluations involve assessment of the clarity and completeness of the description of the analytical procedures, determination of the need for the procedures, and documentation that they have been appropriately validated. Information may vary depending upon the type of method involved. However, in most cases a submission will consist of the following sections.

Published on March 26, 2020
Rationale

This section should identify the need for the procedure and describe the capability of the specific procedure proposed and why it is preferred over other types of determinations. For revised procedures, a comparison should be provided of limitations of the current compendial procedure and advantages offered by the proposed procedure.

Proposed Analytical Procedure

This section should contain a complete description of the analytical procedure sufficiently detailed to enable persons “skilled in the art” to replicate it. The write-up should include all important operational parameters and specific instructions such as preparation of reagents, performance of system suitability tests, description of blanks used, precautions, and explicit formulas for calculation of test results.

Data Elements

This section should provide thorough and complete documentation of the validation of the analytical procedure. It should include summaries of experimental data and calculations substantiating each of the applicable analytical performance characteristics. These characteristics are described in the following section.

VALIDATION

Validation of an analytical procedure is the process by which it is established, by laboratory studies, that the performance characteristics of the procedure meet the requirements for the intended analytical applications. Typical analytical performance characteristics that should be considered in the validation of the types of procedures described in this document are listed in Table 1. Because opinions may differ with respect to terminology and use, each of the performance characteristics is defined in the next section of this chapter, along with a delineation of a typical method or methods by which it may be measured. The definitions refer to “test results”. The description of the analytical procedure should define what the test results for the procedure are. As noted in ISO 5725-1 and 3534-1, a test result is “the value of a characteristic obtained by carrying out a specified test method. The test method should specify that one or a number of individual measurements be made, and their average, or another appropriate function (such as the median or the standard deviation), be reported as the test result. It may also require standard corrections to be applied, such as correction of gas volumes to standard temperature and pressure. Thus, a test result can be a result calculated from several observed values. In the simple case, the test result is the observed value itself.” A test result also can be, but need not be, the final, reportable value that would be compared to the acceptance criteria of a specification. Validation of physical property methods may involve the assessment of chemometric models. However, the typical analytical characteristics used in method validation can be applied to the methods derived from the use of the chemometric models.

<table>
<thead>
<tr>
<th>Table 1. Typical Analytical Characteristics Used in Method Validation</th>
</tr>
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<tbody>
<tr>
<td>Accuracy</td>
</tr>
<tr>
<td>Precision</td>
</tr>
<tr>
<td>Specificity</td>
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<tr>
<td>Detection limit</td>
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<tr>
<td>Quantitation limit</td>
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<td>Linearity</td>
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<td>Range</td>
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<tr>
<td>Robustness</td>
</tr>
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</table>

The effects of processing conditions and potential for segregation of materials should be considered when obtaining a representative sample to be used for validation of procedures.

In the case of compendial procedures, revalidation may be necessary in the following cases: a submission to the USP of a revised analytical procedure or the use of an established general procedure with a new product or raw material (see below in Data Elements Required for Validation).

The ICH documents give guidance on the necessity for revalidation in the following circumstances: changes in the synthesis of the drug substance, changes in the composition of the drug product, and changes in the analytical procedure.

This chapter is intended to provide information that is appropriate to validate a wide range of compendial analytical procedures. The validation of compendial procedures may use some or all of the suggested typical analytical characteristics used in method validation as outlined in Table 1 and categorized by type of analytical method in Table 2. For some compendial procedures the fundamental principles of validation may extend beyond characteristics suggested in this chapter. For these procedures the user is referred to the individual compendial chapter for those specific analytical validation characteristics and any specific validation requirements.
## Analytical Performance Characteristics

### ACCURACY

**Definition:** The accuracy of an analytical procedure is the closeness of test results obtained by that procedure to the true value. The accuracy of an analytical procedure should be established across its range. (A note on terminology: The definition of accuracy in this chapter and ICH Q2 corresponds to unbiasedness only. In the International Vocabulary of Metrology (VIM) and documents of the International Organization for Standardization (ISO), “accuracy” has a different meaning. In ISO, accuracy combines the concepts of unbiasedness (termed “trueness”) and precision.)

**Determination:** In the case of the assay of a drug substance, accuracy may be determined by application of the analytical procedure to an analyte of known purity (e.g., a Reference Standard) or by comparison of the results of the procedure with those of a second, well-characterized procedure, the accuracy of which has been stated or defined.

In the case of the assay of a drug in a formulated product, accuracy may be determined by application of the analytical procedure to synthetic mixtures of the drug product components to which known amounts of analyte have been added within the range of the procedure. If it is not possible to obtain samples of all drug product components, it may be acceptable either to add known quantities of the analyte to the drug product (i.e., “to spike”) or to compare results with those of a second, well-characterized procedure, the accuracy of which has been stated or defined.

In the case of quantitative analysis of impurities, accuracy should be assessed on samples (of drug substance or drug product) spiked with known amounts of impurities. Where it is not possible to obtain samples of certain impurities or degradation products, results should be compared with those obtained by an independent procedure. In the absence of other information, it may be necessary to calculate the amount of an impurity based on comparison of its response to that of the drug substance; the ratio of the responses of equal amounts of the impurity and the drug substance (relative response factor) should be used, if known.

Accuracy is calculated as the percentage of recovery by the assay of the known added amount of analyte in the sample, or as the difference between the mean and the accepted true value, together with confidence intervals.

The ICH documents recommend that accuracy should be assessed using a minimum of nine determinations over a minimum of three concentration levels, covering the specified range (i.e., three concentrations and three replicates of each concentration).

Assessment of accuracy can be accomplished in a variety of ways, including evaluating the recovery of the analyte (percent recovery) across the range of the assay, or evaluating the linearity of the relationship between estimated and actual concentrations. The statistically preferred criterion is that the confidence interval for the slope be contained in an interval around 1.0, or alternatively, that the slope be close to 1.0. In either case, the interval or the definition of closeness should be specified in the validation protocol. The acceptance criterion will depend on the assay and its variability and on the product. Setting an acceptance criterion based on the lack of statistical significance of the test of the null hypothesis that the slope is 1.0 is not an acceptable approach.

Accuracy of physical property methods may be assessed through the analysis of standard reference materials, or alternatively, the suitability of the above approaches may be considered on a case-by-case basis.

### PRECISION

**Definition:** The precision of an analytical procedure is the degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of a homogeneous sample. The precision of an analytical procedure is usually expressed as the standard deviation or relative standard deviation (coefficient of variation) of a series of measurements. Precision may be a measure of either the degree of reproducibility or of repeatability of the analytical procedure under normal operating conditions. In this context, reproducibility refers to the use of the analytical procedure in different laboratories, as in a collaborative study. Intermediate precision (also known as ruggedness) expresses within-laboratory variation, as on different days, or with different analysts or equipment within the same laboratory. Repeatability refers to the use of the analytical procedure within a laboratory over a short period of time using the same analyst with the same equipment.

**Determination:** The precision of an analytical procedure is determined by assaying a sufficient number of aliquots of a homogeneous sample to be able to calculate statistically valid estimates of standard deviation or relative standard deviation (coefficient of variation). Assays in this context are independent analyses of samples that have been carried through the complete analytical procedure from sample preparation to final test result.

The ICH documents recommend that repeatability should be assessed using a minimum of nine determinations covering the specified range for the procedure (i.e., three concentrations and three replicates of each concentration) or using a minimum of six determinations at 100% of the test concentration.

### SPECIFICITY

**Definition:** The ICH documents define specificity as the ability to assess unequivocally the analyte in the presence of components that may be expected to be present, such as impurities, degradation products, and matrix components. Lack of specificity of an individual analytical procedure may be compensated by other supporting analytical procedures. [Note—Other reputable international authorities (UPAC, AOAC-I) have preferred the term “selectivity”, reserving “specificity” for those procedures that are completely selective.] For the tests discussed below, the above definition has the following implications.

**Identification tests:** Ensure the identity of the analyte.

**Purity tests:** Ensure that all of the analytical procedures performed allow an accurate statement of the content of impurities of an analyte (e.g., related substances test, heavy metals limit, or organic volatile impurities).

**Assays:** Provide an exact result, which allows an accurate statement on the content or potency of the analyte in a sample.

**Determination:** In the case of qualitative analyses (identification tests), the ability to select between compounds of closely related structure that are likely to be present should be demonstrated. This should be confirmed by obtaining positive results (perhaps by comparison to a known reference material) from samples containing the analyte, coupled with negative results.
from samples that do not contain the analyte and by confirming that a positive response is not obtained from materials structurally similar to or closely related to the analyte.

In the case of analytical procedures for impurities, specificity may be established by spiking the drug substance or product with appropriate levels of impurities and demonstrating that these impurities are determined with appropriate accuracy and precision.

In the case of the assay, demonstration of specificity requires that it can be shown that the procedure is unaffected by the presence of impurities or excipients. In practice, this can be done by spiking the drug substance or product with appropriate levels of impurities or excipients and demonstrating that the assay result is unaffected by the presence of these extraneous materials.

If impurity or degradation product standards are unavailable, specificity may be demonstrated by comparing the test results of samples containing impurities or degradation products to a second well-characterized procedure (e.g., a pharmacopeial or otherwise validated procedure). These comparisons should include samples stored under relevant stress conditions (e.g., light, heat, humidity, acid/base hydrolysis, and oxidation). In the case of the assay, the results should be compared; in the case of chromatographic impurity tests, the impurity profiles should be compared.

The ICH documents state that when chromatographic procedures are used, representative chromatograms should be presented to demonstrate the degree of selectivity, and peaks should be appropriately labeled. Peak purity tests (e.g., using diode array or mass spectrometry) may be useful to show that the analyte chromatographic peak is not attributable to more than one component.

For validation of specificity for qualitative and quantitative determinations by spectroscopic methods, chapters related to topics such as near-infrared spectrophotometry, Raman spectroscopy, and X-ray powder diffraction should be consulted.

**DETECTION LIMIT**

**Definition:** The detection limit is a characteristic of limit tests. It is the lowest amount of analyte in a sample that can be detected, but not necessarily quantitated, under the stated experimental conditions. Thus, limit tests merely substantiate that the amount of analyte is above or below a certain level. The detection limit is usually expressed as the concentration of analyte (e.g., percentage or parts per billion) in the sample.

**Determination:** For noninstrumental procedures, the detection limit is generally determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected.

For instrumental procedures, the same approach may be used as for noninstrumental procedures. In the case of procedures submitted for consideration as official compendial procedures, it is almost never necessary to determine the actual detection limit. Rather, the detection limit is shown to be sufficiently low by the analysis of samples with known concentrations of analyte above and below the required detection level. For example, if it is required to detect an impurity at the level of 0.1%, it should be demonstrated that the procedure will reliably detect the impurity at that level.

In the case of instrumental analytical procedures that exhibit background noise, the ICH documents describe a common approach, which is to compare measured signals from samples with known low concentrations of analyte with those of blank samples. The minimum concentration at which the analyte can reliably be detected is established. Typically acceptable signal-to-noise ratios are 2:1 or 3:1. Other approaches depend on the determination of the slope of the calibration curve and the standard deviation of responses. Whatever method is used, the detection limit should be subsequently validated by the analysis of a suitable number of samples known to be near, or prepared at, the detection limit.

**QUANTITATION LIMIT**

**Definition:** The quantitation limit is a characteristic of quantitative assays for low levels of compounds in sample matrices, such as impurities in bulk drug substances and degradation products in finished pharmaceuticals. It is the lowest amount of analyte in a sample that can be determined with acceptable **Precision** and **Accuracy** under the stated experimental conditions. The quantitation limit is expressed as the concentration of analyte (e.g., percentage or parts per billion) in the sample.

**Determination:** For noninstrumental procedures, the quantitation limit is generally determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be determined with acceptable **Accuracy** and **Precision**.

For instrumental procedures, the same approach may be used as for noninstrumental procedures. In the case of procedures submitted for consideration as official compendial procedures, it is almost never necessary to determine the actual quantitation limit. Rather, the quantitation limit is shown to be sufficiently low by the analysis of samples with known concentrations of analyte above and below the quantitation level. For example, if it is required that an analyte be assayed at the level of 0.1 mg/tablet, it should be demonstrated that the procedure will reliably quantitate the analyte at that level.

In the case of instrumental analytical procedures that exhibit background noise, the ICH documents describe a common approach, which is to compare measured signals from samples with known low concentrations of analyte with those of blank samples. The minimum concentration at which the analyte can reliably be quantified is established. A typically acceptable signal-to-noise ratio is 10:1. Other approaches depend on the determination of the slope of the calibration curve and the standard deviation of responses. Whatever approach is used, the quantitation limit should be subsequently validated by the analysis of a suitable number of samples known to be near, or prepared at, the quantitation limit.

**LINEARITY AND RANGE**

**Definition of linearity:** The linearity of an analytical procedure is its ability to elicit test results that are directly, or by a well-defined mathematical transformation, proportional to the concentration of analyte in samples within a given range. Thus, in this section, “linearity” refers to the linearity of the relationship of concentration and assay measurement. In some cases, to attain linearity, the concentration and/or the measurement may be transformed. [Note—The weighting factors used in the regression analysis may change when a transformation is applied.] Possible transformations may include log, square root, or
reciprocal, although other transformations are acceptable. If linearity is not attainable, a nonlinear model may be used. The goal is to have a model, whether linear or nonlinear, that describes closely the concentration–response relationship.

Definition of range: The range of an analytical procedure is the interval between the upper and lower levels of analyte (including these levels) that have been demonstrated to be determined with a suitable level of precision, accuracy, and linearity using the procedure as written. The range is normally expressed in the same units as test results (e.g., percent or parts per million) obtained by the analytical procedure.

**Determination of linearity and range:** Linearity should be established across the range of the analytical procedure. It should be established initially by visual examination of a plot of signals as a function of analyte concentration of content. If there appears to be a linear relationship, test results should be established by appropriate statistical methods (e.g., by calculation of a regression line by the method of least squares). Data from the regression line itself may be helpful to provide mathematical estimates of the degree of linearity. The correlation coefficient, y-intercept, slope of the regression line, and residual sum of squares should be submitted.

The range of the procedure is validated by verifying that the analytical procedure provides acceptable precision, accuracy, and linearity when applied to samples containing analyte at the extremes of the range as well as within the range.

ICH recommends that, for the establishment of linearity, a minimum of five concentrations normally be used. It is also recommended that the following minimum specified ranges should be considered:

- **Assay of a drug substance (or a finished product):** From 80% to 120% of the test concentration
- **Determination of an impurity:** From 50% to 120% of the acceptance criterion
- **For content uniformity:** A minimum of 70%–130% of the test concentration, unless a wider or more appropriate range based on the nature of the dosage form (e.g., metered-dose inhalers) is justified
- **For dissolution testing:** ±20% over the specified range (e.g., if the acceptance criteria for a controlled-release product cover a region from 30% after 1 h, and up to 90% after 24 h, the validated range would be 10%–110% of the label claim).

The traditional definition of linearity, i.e., the establishment of a linear or mathematical relationship between sample concentration and response, is not applicable to particle size analysis. For particle size analysis, a concentration range is defined (instrument- and particle size-dependent) such that the measured particle size distribution is not affected by changes in concentration within the defined concentration range. Concentrations below the defined concentration range may introduce an error due to poor signal-to-noise ratio, and concentrations exceeding the defined concentration range may introduce an error due to multiple scattering.

**ROBUSTNESS**

**Definition:** The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in procedural parameters listed in the procedure documentation and provides an indication of its suitability during normal usage. Robustness may be determined during development of the analytical procedure.

**SYSTEM SUITABILITY**

If measurements are susceptible to variations in analytical conditions, these should be suitably controlled, or a precautionary statement should be included in the procedure. One consequence of the evaluation of Robustness and ruggedness should be that a series of system suitability parameters is established to ensure that the validity of the analytical procedure is maintained whenever used. Typical variations are the stability of analytical solutions, different equipment, and different analysts. In the case of liquid chromatography, typical variations are the pH of the mobile phase, the mobile phase composition, different lots or suppliers of columns, the temperature, and the flow rate. In the case of gas chromatography, typical variations are different lots or suppliers of columns, the temperature, and the flow rate.

System suitability tests are based on the concept that the equipment, electronics, analytical operations, and samples to be analyzed constitute an integral system that can be evaluated as such. System suitability test parameters to be established for a particular procedure depend on the type of procedure being evaluated. They are especially important in the case of chromatographic procedures. Submissions to the USP should make note of the requirements in Chromatography (621), System Suitability.

**Data Elements Required for Validation**

Compendial test requirements vary from highly exacting analytical determinations to subjective evaluation of attributes. Considering this broad variety, it is only logical that different test procedures require different validation schemes. This chapter covers only the most common categories of tests for which validation data should be required. These categories are as follows:

**CATEGORY I**

Analytical procedures for quantitation of major components of bulk drug substances or active ingredients (including preservatives) in finished pharmaceutical products.

**CATEGORY II**

Analytical procedures for determination of impurities in bulk drug substances or degradation compounds in finished pharmaceutical products. These procedures include quantitative assays and limit tests.

**CATEGORY III**

Analytical procedures for determination of performance characteristics (e.g., dissolution, drug release, and others).
### CATEGORY IV

Identification tests,

For each category, different analytical information is needed. Listed in Table 2 are data elements that are normally required for each of these categories. [Note—For detailed information regarding the validation of dissolution procedures, see The Dissolution Procedure: Development and Validation (1092).]

<table>
<thead>
<tr>
<th>Analytical Performance Characteristics</th>
<th>Category I</th>
<th>Category II</th>
<th>Category III</th>
<th>Category IV</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Quantitative</td>
<td>Limit Tests</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Accuracy</td>
<td>Yes</td>
<td>*</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Precision</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Specificity</td>
<td>Yes</td>
<td>Yes</td>
<td>*</td>
<td>Yes</td>
</tr>
<tr>
<td>Detection limit</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Quantitation limit</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Linearity</td>
<td>Yes</td>
<td>No</td>
<td>*</td>
<td>No</td>
</tr>
<tr>
<td>Range</td>
<td>Yes</td>
<td>Yes</td>
<td>*</td>
<td>No</td>
</tr>
</tbody>
</table>

*a May be required, depending on the nature of the specific test.

Already established general procedures (e.g., titrimetric determination of water or bacterial endotoxins) should be verified to establish their suitability for use, such as their accuracy (and absence of possible interference) when used for a new product or raw material.

When validating physical property methods, consider the same performance characteristics required for any analytical procedure. Evaluate use of the performance characteristics on a case-by-case basis, with the goal of determining that the procedure is suitable for its intended use. The specific acceptance criteria for each validation parameter should be consistent with the intended use of the method.

Physical methods may also be classified into the four validation categories. For example, validation of a quantitative spectroscopic method may involve evaluation of Category I or Category II Analytical Performance Characteristics, depending on the method requirements. Qualitative physical property measurements, such as particle size, surface area, bulk and tapped density, which could impact performance characteristics, often best fit in Category III. Category IV Analytical Performance Characteristics usually applies to validation of qualitative identification spectroscopic methods. However, the various techniques may be used for different purposes, and the specific use of the method and characteristics of the material being analyzed should be considered when definitively applying a category to a particular type of method.

The validity of an analytical procedure can be verified only by laboratory studies. Therefore, documentation of the successful completion of such studies is a basic requirement for determining whether a procedure is suitable for its intended application(s). Current compendial procedures are also subject to regulations that require demonstration of suitability under actual conditions of use (see Verification of Compendial Procedures (1226) for principles relative to the verification of compendial procedures). Appropriate documentation should accompany any proposal for new or revised compendial analytical procedures.

1226 VERIFICATION OF COMPENDIAL PROCEDURES

The intent of this chapter is to provide general information on the verification of compendial procedures that are being performed for the first time to yield acceptable results utilizing the personnel, equipment, and reagents available. This chapter is not intended for retroactive application to already successfully established laboratory procedures. Validation of Compendial Procedures (1225) provides general information on characteristics that should be considered for various test categories and on the documentation that should accompany analytical procedures submitted for inclusion in USP–NF. Verification consists of assessing selected analytical performance characteristics, such as those that are described in (1225), to generate appropriate, relevant data rather than repeating the validation process.

Users of compendial analytical procedures are not required to validate these procedures when first used in their laboratories, but documented evidence of suitability should be established under actual conditions of use. In the United States, this requirement is established in 21 CFR 211.194(a)(2) of the current Good Manufacturing Practice regulations, which states that the “suitability of all testing methods used shall be verified under actual conditions of use.”

Verification of microbiological procedures is not covered in this chapter because it is covered in Antimicrobial Effectiveness Testing (51), Microbial Enumeration Tests (61), Tests for Specified Microorganisms (62), Sterility Tests (71), and Validation of Microbial Recovery from Pharmacopeial Articles (1227).
**Change to read:**

**VERIFICATION PROCESS**

The verification process for compendial test procedures is the assessment of whether the procedure can be used for its intended purpose, under the actual conditions of use for a specified drug substance and/or drug product matrix.

Users should have the appropriate experience, knowledge, and training to understand and be able to perform the compendial procedures as written. Verification should be conducted by the user such that the results will provide confidence that the compendial procedure will perform suitably as intended.

If the verification of the compendial procedure is not successful, and assistance from USP staff has not resolved the problem, it may be concluded that the procedure may not be suitable for use with the article being tested in that laboratory. It may then be necessary to develop and validate an alternative procedure as allowed in the General Notices, 6.30 Alternative and Harmonized Methods and Procedures. The alternative procedure may be submitted to USP, along with the appropriate data, to support a proposal for inclusion or replacement of the current compendial procedure.

**VERIFICATION REQUIREMENTS**

Verification requirements should be based on an assessment of the complexity of both the procedure and the material to which the procedure is applied. Although complete revalidation of a compendial method is not required to verify the suitability of a procedure under actual conditions of use, some of the analytical performance characteristics listed in Validation of Compendial Procedures (1225), Table 2, may be used for the verification process. Only those characteristics that are considered to be appropriate for the verification of the particular procedure need to be evaluated. The process of assessing the suitability of a compendial analytical test procedure under the conditions of actual use may or may not require actual laboratory performance of each analytical performance characteristic. The degree and extent of the verification process may depend on the level of training and experience of the user, on the type of procedure and its associated equipment or instrumentation, on the specific procedural steps, and on which article(s) are being tested.

Verification should assess whether the compendial procedure is suitable for the drug substance and/or the drug product matrix, taking into account the drug substance’s synthetic route, the method of manufacture for the drug product, or both, if applicable. Verification should include an assessment of elements such as the effect of the matrix on the recovery of impurities and drug substances from the drug product matrix, as well as the suitability of chromatographic conditions and column, the appropriateness of detector signal response, etc.

As an example, an assessment of specificity is a key parameter in verifying that a compendial procedure is suitable for use in assaying drug substances and drug products. For instance, acceptable specificity for a chromatographic method may be verified by conformance with system suitability resolution requirements (if specified in the procedure). However, drug substances from different suppliers may have different impurity profiles that are not addressed by the compendial test procedure. Similarly, the excipients in a drug product can vary widely among manufacturers and may have the potential to directly interfere with the procedure or cause the formation of impurities that are not addressed by the compendial procedure. In addition, drug products containing different excipients, antioxidants, buffers, or container extractives may affect the recovery of the drug substance from the matrix. In these cases, a more thorough assessment of the matrix effects may be required to demonstrate suitability of the procedure for the particular drug substance or product. Other analytical performance characteristics such as an assessment of the limit of detection or quantitation and precision for impurities procedures may be useful to demonstrate the suitability of the compendial procedure under actual conditions of use. It is the user’s responsibility to demonstrate the long term (more than 24 h) stability and storage conditions of Standard and sample preparations throughout the duration of the procedure during the verification of compendial procedures.

Verification is not required for basic compendial test procedures that are routinely performed unless there is an indication that the compendial procedure is not appropriate for the article under test. Examples of basic compendial procedures include, but are not limited to, loss on drying, residue on ignition, various wet chemical procedures such as acid value, and simple instrumental determinations such as pH measurements. However, for the application of already established routine procedures to compendial articles tested for the first time, it is recommended that consideration be given to any new or different sample handling or solution preparation requirements.

**〈1227〉 VALIDATION OF MICROBIAL RECOVERY FROM PHARMACOPEIAL ARTICLES**

**INTRODUCTION**

This chapter provides guidelines for the validation of recovery methods for the estimation of the number of viable microorganisms, the detection of indicators or specified microorganisms, and the sterility testing of pharmacopeial articles. The test procedures in Antimicrobial Effectiveness Testing (51), Sterility Tests (71), Microbial Enumeration Tests (61), and Tests for Specified Microorganisms (62) are considered validated. However, use of compendial methods requires establishment of...
suitability of the method demonstrating recovery of the challenge organisms in the presence of the product. Alternatives/ modifications to these recovery procedures beyond what are described in these chapters (such as dilution, chemical or enzymatic neutralization, and membrane filtration) require validation. It is generally understood that if a product possesses antimicrobial properties because of the presence of a specific preservative or because of its formulation, this antimicrobial property must be neutralized to recover viable microorganisms. This neutralization may be achieved by the use of a specific neutralizer, by dilution, by a combination of dilution, filtration, and rinsing, or by any combination of these methods. When the product displays intrinsic antimicrobial activity for a given microorganism and, given this antimicrobial activity, the risk of microbial contamination is low, the method could be considered as fit for the purpose of providing a strong rationale.

**Change to read:**

### INFLUENTIAL FACTORS

Several factors affect the measurement of a test solution’s antimicrobial activity, and these must be considered in the validation design. They include the nature of the microorganisms used as challenge organisms, preparation of the inoculum of challenge organisms, specific conditions of the test, and conditions of recovery. These factors also affect the validation of recovery methods for aqueous or nonaqueous products, irrespective of their antimicrobial properties; thus, all test methods should be validated with these factors in mind.

The nature of the challenge microorganism exerts a strong effect upon the response to the antimicrobial agent, and so upon the neutralization required for recovery. Represented among these organisms in compendial tests are gram-positive bacteria, gram-negative bacteria, anaerobic bacteria, yeasts, and molds. Each organism to be used in the test must be included in the validation.

The preparation of the inoculum of challenge microorganisms also affects the testing of products having antimicrobial properties. The growth and preparation of the challenge organism determines the physiological state of the cell. This state has a direct influence on the results of any test of antimicrobial efficacy. Microbial tests do not use individual cells; rather, populations of cells are harvested for study. The data generated from these studies are less variable if the cell populations are homogeneous. Liquid cultures or confluent growths on solid medium are best suited for reproducible culture preparation. The conditions of organism preparation and storage must be standardized for the neutralizer evaluation and should reflect the conditions of the antimicrobial assay.

The specific conditions of the test, including buffers used, water, light conditions, and temperature, must be reproduced in the validation study. All test conditions also should be standardized and performed in the validation study exactly as performed in the test.

The conditions of microbial recovery are among the most crucial in accurately estimating the number of microorganisms present in a test solution. The first consideration is the recovery medium used to support the growth of survivors. This concern is discussed in detail below. The second consideration is the incubation conditions. Optimal conditions for growth must be present to ensure complete growth and reproducible results.

**Change to read:**

### METHODS OF NEUTRALIZING ANTIMICROBIAL PROPERTIES

Three common methods are used to neutralize antimicrobial properties of a product: 1) chemical inhibition, 2) dilution, and 3) filtration and rinsing.

**Chemical Neutralization**

Table 1 shows known neutralizers for a variety of chemical antimicrobial agents and the reported toxicity of some chemical neutralizers to specific microorganisms. However, despite potential toxicity, the convenience and quick action of chemical inhibitors encourage their use. Chemical neutralization of antimicrobial agents is the preferred method for the antimicrobial efficacy test. The potential of chemical neutralizers should be considered in the membrane filtration and the direct inoculation sterility tests. Antibiotics may not be susceptible to neutralization by chemical means, but rather by enzymatic treatment (e.g., penicillinase). These enzymes may be used where required.

<table>
<thead>
<tr>
<th>Neutralizer</th>
<th>Antimicrobial Agents</th>
<th>Class</th>
<th>Potential Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bisulfate</td>
<td>Glutaraldehyde, mercurials</td>
<td>Non-spore forming bacteria</td>
<td></td>
</tr>
<tr>
<td>Dilution</td>
<td>Phenolics, alcohol, aldehydes, sorbate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycine</td>
<td>Aldehydes</td>
<td>Growing cells</td>
<td></td>
</tr>
<tr>
<td>Lecithin</td>
<td>Quaternary ammonium compounds (QACs), para-bens, bis-biguanides</td>
<td></td>
<td>Bacteria</td>
</tr>
<tr>
<td>Mg²⁺ or Ca²⁺ ions</td>
<td>EDTA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Polysorbate</td>
<td>QACS, iodoine, parabens</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Published on March 26, 2020
Dilution

A second approach to neutralizing antimicrobial properties of a product is by dilution, because the concentration of a chemical antimicrobial agent exerts a large effect on its potency. The relationship between concentration and antimicrobial effect differs among bactericidal agents but is constant for a particular antimicrobial agent. This relationship is exponential in nature, with the general formula:

\[ C^\eta t = k \]

- \( C \) = concentration of the antimicrobial agent
- \( \eta \) = concentration exponent (dilution coefficient), the slope of the plot of log \( t \) versus log \( C \)
- \( t \) = time required to kill a standard inoculum
- \( k \) = a constant

Antimicrobial agents with high \( \eta \) values are rapidly neutralized by dilution, whereas those with low \( \eta \) values are not good candidates for neutralization by dilution (see Table 2).

### Table 2. Concentration Exponents for Some Common Antimicrobial Agents

<table>
<thead>
<tr>
<th>Representative Antimicrobial Agent</th>
<th>( \eta ) Values</th>
<th>Increased Time Factor (x) to Kill Microorganisms When the Concentration is Reduced to:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>One-Half</td>
</tr>
<tr>
<td>Phenolics</td>
<td>6</td>
<td>64</td>
</tr>
<tr>
<td>Alcohol</td>
<td>10</td>
<td>1024</td>
</tr>
<tr>
<td>Parabens</td>
<td>2.5</td>
<td>6</td>
</tr>
<tr>
<td>Chlorhexidine</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Mercury compounds</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Quaternary ammonium compounds</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

Membrane Filtration

An approach that is often used, especially in sterility testing, is neutralization by membrane filtration. This approach relies upon the physical retention of the microorganism on the membrane filter, with the antimicrobial agent passing through the filter into the filtrate. The filter is then incubated for recovery of viable microorganisms. However, filtration alone may not remove sufficient quantities of the antimicrobial agent to allow growth of surviving microorganisms. Adherence of residual antimicrobial agents to the filter membrane may cause growth inhibition. Filtration through a low-binding filter material, such as polyvinylidene difluoride, helps to minimize this growth inhibition. Additionally, the preservative may be diluted or flushed from the filter by rinsing with a non-toxic fluid, such as diluting Fluid A (see Sterility Tests (71), Diluting and Rinsing Fluids for Membrane Filtration for diluting fluid compositions). Chemical neutralizers in the rinsing fluid can ensure that any antimicrobial residue on the membrane does not interfere with the recovery of viable microorganisms.

Change to read:

**VALIDATION OF NEUTRALIZATION METHODS—RECOVERY COMPARISONS**

A validated method for neutralizing the antimicrobial properties of a product must meet two criteria: neutralizer efficacy and neutralizer non-toxicity. The validation study documents that the neutralization method employed is effective in inhibiting the antimicrobial properties of the product (neutralizer efficacy) without impairing the recovery of viable microorganisms (lack of neutralizer toxicity). Validation protocols may meet these two criteria by comparing recovery results for treatment groups.

The first is the test group, in which the product is subjected to the neutralization method, then a low level of challenge microorganism (less than 100 colony-forming units (cfu)) is inoculated for recovery. The second is the peptone control group, in which the neutralization method is used with peptone, or diluting Fluid A (see Sterility Tests (71), Diluting and Rinsing Fluids for Membrane Filtration), as the test solution. The third is the viability group, in which the actual inoculum is used without exposure to the neutralization scheme. Similar recovery between the test group and the peptone group demonstrates adequate
neutralizer efficacy; similar recovery between the peptone group and the viability group demonstrates adequate neutralizer non-toxicity. In principle, the protocol must show that recovery of a low inoculum (less than 100 cfu) is not inhibited by the test sample and the neutralization method. Validation protocols may meet these two criteria by comparing recovery among three distinct test groups: 1) neutralized product with inoculum, 2) challenge inoculum control in buffered solution, and 3) inoculum in the absence of product or neutralizer. This can be established by directly comparing the result in the treated solution (1) to the inoculum (3) above. If the growth on the treated solution is not comparable to the growth on the inoculum group, it should be determined whether the neutralization method itself is toxic to the microorganisms.

**Recovery on Agar Medium**

In the tests under (51) and (61), the number of viable challenge microorganisms in the product is estimated by calculating the concentration of cfu per milliliter by the plate count method. A design for validating neutralization would incorporate the treatment groups as described under Validation of Neutralization Methods—Recovery Comparisons. At least three independent replicates of the experiment should be performed, and each should demonstrate a mean count of any of the test organisms not differing by a factor greater than 2, i.e., 50%–200% recovery, from the value of the control in the absence of product. If it is necessary to solubilize the test sample, the effects of the solubilization method on viable microorganisms must be determined. This situation can occur when testing ointments, suspensions, or other articles.

If a greater number of replicates is required in the validation study, the comparisons may be evaluated by transforming the numbers of cfu to their logarithmic values and analyzing the data statistically by the Student t test (pairwise comparisons) or by analysis of variance (ANOVA) for comparing all groups. If ANOVA is used, and significant differences among the populations are determined, a test such as Dunnett’s test may be used, with the peptone group used as the control group.

**Recovery by Membrane Filtration**

This validation follows the procedure described in Sterility Tests (71), Method Suitability Test, with the exception of plating on solid medium to quantitate recovery. It should be emphasized that quantitative recovery is not required to demonstrate sterility test suitability. It only requires a qualitative assessment (visual turbidity). Three 100-mL rinses are assumed, but the volume and number of rinses are subject to validation. A maximum of five 100-mL washes should be used for routine testing even if during method suitability it has been demonstrated that such a cycle does not fully eliminate the antimicrobial activity. Each validation run should be performed independently at least three times.

In the test solution group, the product is passed through the membrane filter, followed by two 100-mL portions of diluting-neutralizing fluid. After the second rinse has been filtered, a final 100-mL portion containing less than 100 cfu of the specific challenge microorganism is passed through the filter. This filter is then placed on the appropriate agar recovery medium and incubated for recovery.

The inoculum is directly plated onto the solid medium. It is possible that filtration will lead to reduced recovery of the challenge microorganism, either through inherent toxicity of the membrane or by adherence of the microorganism to the filtration vessel walls. A control group can be used to evaluate this component of membrane filtration validation. Diluting Fluid A is used as the dilution medium without exposing the filter to the product. After addition of the low-level inoculum to the final rinse, the filter is plated as above. Technique-specific loss of microorganisms can be estimated by comparing the recovery in the diluting Fluid A (see Sterility Tests, Diluting and Rinsing Fluids for Membrane Filtration) group to the inoculum count.

It is assumed in this discussion that the test sample can be filtered. If it is necessary to solubilize the test sample, the effects of the solubilization method on viable microorganisms must be determined. This situation can occur when testing ointments, suspensions, or other articles.

The method can be considered validated if the recovery rate in the three independent replicates is similar for the test solution and the diluting Fluid A (see Sterility Tests, Diluting and Rinsing Fluids for Membrane Filtration) control.

**Recovery in Liquid Medium**

It is assumed in Sterility Tests, Test for Sterility of the Product to be Examined, Direct Inoculation of the Culture Medium that the recovery medium will allow for growth of all surviving microorganisms. The broth in that test must serve both to neutralize any antimicrobial properties of the test solution and to support the growth of the microorganisms. The treatment groups described under Validation of Neutralization Methods—Recovery Comparisons can be used for validation of the recovery method, with the proportions of product and recovery medium varied to achieve adequate neutralization. The method can be considered validated if all groups show clearly visible growth visually comparable to that in the control vessel without product within the indicated time period in.

**RECOVERY OF INJURED MICROORGANISMS**

The validation studies described above use challenge microorganisms that have never been exposed to antimicrobial agents, and thus are not identical to organisms seen in antimicrobial effectiveness testing or when a sterility test is performed on a preserved product. If the use of alternative media is desired, the recovery of injured microorganisms should be addressed in the validation study. This may be done by directly comparing the recovery of each challenge microorganism on the preferred medium and on the alternative medium, after exposure to the product. This exposure should include at least two time periods showing survival of less than 100 cfu/mL, unless the rate of kill of the antimicrobial agent is such that no recovery is possible.
even if the microorganism is plated within minutes of exposure. This comparison should be performed at least three times. The alternative medium is validated if the recovery seen on that medium is no less than that seen on the preferred medium, within an error of 0.5 log units.

Change to read:

**ESTIMATING THE NUMBER OF COLONY-FORMING UNITS**

The accuracy of any estimate of viable cfu is affected by the number plated. As the number of viable cells plated increases, crowding effects decrease the accuracy of the count, reducing the estimate. As the number decreases, random error plays an increasing role in the estimate.

The accepted range for countable colonies on a standard agar plate is between 25 and 250 for most bacteria and *Candida albicans*. This range was established in the food industry for counting coliform bacteria in milk. This range is acceptable for compendial organisms, except for fungi. It is not optimal for counting all environmental isolates. The recommended counting range for *Aspergillus brasiliensis* is between 8 and 80 cfu/plate.

Lower counting thresholds for the greatest dilution plating in series must be justified. Numbers of colonies on a plate follow the Poisson distribution, so the variance of the mean value equals the mean value of counts. Therefore, as the mean number of cfu per plate becomes lower, the percentage error of the estimate increases (see Table 3). For example, 3 cfu/plate at the $10^{-1}$ dilution provide an estimate of 30 cfu/mL, with an error of 58% of the estimate.

<table>
<thead>
<tr>
<th>cfu/Plate</th>
<th>Standard Error</th>
<th>Error as % of Mean</th>
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<tbody>
<tr>
<td>30</td>
<td>5.48</td>
<td>18.3</td>
</tr>
<tr>
<td>29</td>
<td>5.39</td>
<td>18.6</td>
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<td>5.29</td>
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<td>20.4</td>
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<td>23</td>
<td>4.80</td>
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<td>4.69</td>
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</tr>
<tr>
<td>2</td>
<td>1.41</td>
<td>70.7</td>
</tr>
</tbody>
</table>

*Table 3. Error as a Percentage of Mean for Plate Counts*
<1231> WATER FOR PHARMACEUTICAL PURPOSES

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1. INTRODUCTION

Water is widely used as a raw material, inactive ingredient, medicinal vehicle, and solvent in the processing, formulation, and manufacture of pharmaceutical products (dosage forms), active pharmaceutical ingredients (APIs), API intermediates, compendial articles, and analytical reagents as well as in cleaning applications.

This is an informational chapter on pharmaceutical water topics and includes some of the chemical and microbiological concerns unique to water and its preparation and uses. The chapter provides information about water quality attributes (that may or may not be included within a water monograph) and processing techniques that can be used to improve water quality. It also discusses water system validation and gives a description of minimum water quality standards that should be considered when selecting a water source including sampling and system controls. It is equally important for water systems to be operated and maintained in a state of control to provide assurance of operational stability and therefore the capability to provide water that meets established water quality standards.

This informational chapter is intended to be educational, and the user should also refer to existing regulations or guidelines that cover U.S. and international [International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) or World Health Organization (WHO)] good manufacturing practice (GMP) issues, as well as operational and engineering guides and/or other regulatory guidance for water [e.g., from the Food and Drug Administration (FDA), Environmental Protection Agency (EPA), or WHO]. This chapter is not, and should not be considered, an all-inclusive document on pharmaceutical waters. It contains basic information and points to be considered for the processing, holding, monitoring, and use of water. It is the user's responsibility to ensure that:

1. The selection of the type and specifications of water is appropriate for its intended use.
2. Water production and quality meet applicable governmental regulations and guidance.
3. The pharmacopeial specifications for the types of water used in monographed articles are met.
4. Water used in the preparation of reagents for analysis or the performance of required tests meets USP requirements.

Control and monitoring of the chemical and endotoxin purity of waters is important for complying with the requirements of the monographs in this compendium. Attributes listed in USP monographs should be considered the "minimum" requirements. More stringent requirements may be needed for some applications to ensure suitability for particular uses. Basic guidance on the appropriate applications of waters can be found in the monographs and is also discussed further in this chapter.

Control of the microbiological quality of water is also important for many of its uses. This attribute is intentionally not specified in most water monographs. Microbiological control is discussed throughout this chapter, but especially in sections 4. Validation and Qualification of Water Purification, Storage, and Distribution Systems, 5. Design and Operation of Purified Water and Water for Injection Systems, 6. Sampling, 8. Microbial Evaluations, and 9. Alert and Action Levels and Specifications.

This chapter contains various chemical, microbiological, processing, and engineering concepts of importance to users of water. Water system validation, process control levels, and specifications are also presented later in this chapter.

2. SOURCE WATER CONSIDERATIONS

Source water is the water that enters the facility. The origin of this source water can be from natural surface waters like rivers and reservoirs, deep-bed well waters, sea waters, or some combination of these, potentially including multiple locations of each type of source water. Thus, source water can be supplied from these various origins (public or private), from municipalities’ on-site water sourcing, or by external delivery such as a truck. It is possible that source water may not be potable and safe to drink. Such water may require pretreatment to ensure that it meets drinking water standards. It is the responsibility of the users of any source water to ensure that the water used in the production of drug substances (API), as well as water for indirect drug product contact or for purification system feed water purposes meets, at a minimum, drinking (potable) water standards as defined by the requirements of the National Primary Drinking Water Regulations (NPDWR) (40 CFR 141) issued by the U.S. EPA.
or the drinking water regulations of the European Union (EU) or Japan, or the WHO drinking water guidelines (see 3.3.1 Drinking Water). These regulations establish limits on the types and quantities of certain chemical and microbiological contaminants and ensure that the water will contain safe quantities of chemical and microbial species.

Where water supplies are from regulated water utility companies, less stringent monitoring may be possible because the attributes may be tested regularly and ensured by the supplier (see 9.4.5 Source Water Control). Water being withdrawn from a nonregulated supply should be sampled and tested appropriately at a suitable frequency that takes into account local environmental and seasonal changes and other quality fluctuations. Testing should ensure conformance with one of the drinking water standards discussed above.

The use of water complying with one of these designated drinking waters as a source water allows water pretreatment systems to only be challenged to remove small quantities of potentially difficult-to-remove chemicals. Control of objectionable chemical contaminants at the source water stage eliminates the need to specifically test for some of them [e.g., trihalomethanes and elemental impurities (see Elemental Impurities—Limits (232))] after the water has been further purified, assuming there is no opportunity for recontamination.

Source waters can be used for nonproduct contact purposes such as for non-contact cooling systems. Such water may not normally be required to meet drinking water standards. Under such circumstances, the quality standards for this water when used in a pharmaceutical facility should be subject to quality standards established by the user and defensible to regulatory agencies.

3. WATERS USED FOR PHARMACEUTICAL MANUFACTURING AND TESTING PURPOSES

There are many different grades of water used for pharmaceutical purposes. Several are described in USP monographs that specify uses, acceptable methods of preparation, and quality attributes. These waters can be divided into two general types: bulk waters, which are typically produced on-site where they are used; and sterile waters, which are produced, packaged, and sterilized to preserve microbial quality throughout their packaged shelf life. There are several specialized types of sterile waters that differ in their designated applications, packaging limitations, and other quality attributes. Monographed waters must meet the quality attributes as specified in the related monographs, and any Notes appearing in those monographs should be considered and addressed.

With the exception of Bacteriostatic Water for Injection, the monographed bulk and sterile waters have a statement indicating that there are no added substances, or no added antimicrobial agents. In the case of antimicrobial agents, the purpose is to ensure that the sterile water product is rendered sterile based solely on its preparation, packaging, and storage. In the case of the more general statement, “no added substances”, this requirement is intended to mean “no added substances that aren’t sufficiently removed”. Two specific examples support this intention, but there are many examples. First, the use of softeners is commonplace. A softener replaces calcium and magnesium ions (also known as hardness ions) with sodium, so technically you are adding two sodium ions for each hard ion. The purpose of sodium displacement is to protect downstream equipment from the hard water. The sodium ions are eventually removed sufficiently, and this is proven when the water sample passes the test in Water Conductivity (645). Another specific example is the use of ozone as a sanitant that is added to the storage tank for microbial control. This could be considered an added substance, unless the ozone is destroyed before use, as is normally the case. Other notable examples include the addition of chlorine to kill bacteria in the pretreatment system, use of bisulfite to chemically reduce chlorine to chloride and protect downstream equipment, and use of a nitrogen blanket for protection from atmospheric contamination.

There are also other types of water for which there are no monographs. These are waters with names given for descriptive purposes only. Many of these waters are used in specific analytical methods. The descriptive titles may imply certain quality attributes or modes of preparation, but these nonmonographed waters may not necessarily adhere strictly to the stated or implied modes of preparation or specified attributes. Waters produced by other means or controlled by other test attributes, or even a monographed water, may equally satisfy the intended uses for these waters. It is the user’s responsibility to ensure that such waters, even if produced and controlled exactly as stated, are suitable for their intended use. Wherever the term “water” is used within this compendium without other descriptive adjectives or clauses, the intent is that water of no less purity than USP Purified Water be used (see 3.1.1 Purified Water). A brief description of the various types of waters commonly associated with pharmaceutical applications and their significant uses or attributes follows.

Figure 1 may be helpful in understanding some of the various types of waters, their preparation, and uses.
3.1 Bulk Monographed Waters and Steam

The following waters are generally produced in large volumes using a multiple-unit operation water system. These waters are typically distributed in a piping system for use at the same site.

3.1.1 PURIFIED WATER

Purified Water (see the USP monograph) is used as an excipient in the production of nonparenteral preparations and in other pharmaceutical applications, such as the cleaning of nonparenteral product-contact components and equipment. Unless otherwise specified, Purified Water is also to be used as the minimum water quality for all tests and assays in which “water” is indicated (see General Notices, 8.230.30 Water in a Compendial Procedure). This applies regardless of the font and letter case used in its spelling.

The minimal quality of source water for the production of Purified Water is Drinking Water whose attributes are prescribed by the U.S. EPA, EU, Japan, or WHO. This source water may be purified using unit operations that include deionization, distillation, ion exchange, reverse osmosis, filtration, or other suitable purification procedures. Purified Water must meet the requirements for ionic and organic chemical purity and must be protected from microbial contamination. Purified Water systems must be validated to reliably and consistently produce and distribute water of acceptable chemical and microbiological quality. Purified Water systems that function under ambient conditions are particularly susceptible to the establishment of biofilms of microorganisms, which can be the source of undesirable levels of viable microorganisms or endotoxins in the water. These ambient Purified Water systems require frequent sanitization and microbiological monitoring to ensure that the water reaching the points of use has appropriate microbiological quality.

The Purified Water monograph also allows bulk packaging for commercial use elsewhere. In contrast to Sterile Purified Water, packaged Purified Water is not required to be sterile. Because there is potential for microbial contamination and other quality changes in this packaged nonsterile water, this form of Purified Water should be prepared and stored in a manner that limits microbial growth, and/or should be used in a timely fashion before microbial proliferation renders it unsuitable for its intended use. Also, depending on the material used for packaging, extractable compounds could be leaching into the water from the packaging. Although this article is required to meet the same chemical purity standards as the bulk water, extractables from the packaging will likely render the packaged water less chemically pure than the bulk water. The nature of these impurities may even render the water an inappropriate choice for some applications. It is the user’s responsibility to ensure fitness for use of this packaged article when it is used in manufacturing, clinical, or analytical applications where the purer bulk form of the water is indicated.

3.1.2 WATER FOR INJECTION

Water for Injection (see the USP monograph) is used as an excipient in the production of parenteral and other preparations where product endotoxin content must be controlled, and in other pharmaceutical applications, such as the cleaning of certain equipment and parenteral product-contact components.

The minimal quality of source water for the production of Water for Injection is Drinking Water whose attributes are prescribed by the U.S. EPA, EU, Japan, or WHO. This source water may be treated to render it suitable for subsequent final purification steps, such as distillation (or whatever other validated process is used, according to the monograph). The finished water must meet all of the chemical requirements specified in the monograph, as well as an additional bacterial endotoxin specification. Because endotoxins are produced by the kinds of microorganisms that are prone to inhabit water systems, the equipment and procedures used by the system to purify, store, and distribute Water for Injection should be designed to control microbial contamination and must be designed to remove incoming endotoxins from the source water. Water for Injection systems must be validated to reliably and consistently produce and distribute this quality of water.
The Water for Injection monograph also allows bulk packaging for commercial use. In contrast to Sterile Water for Injection, packaged Water for Injection is not required to be sterile. However, to preclude significant changes in its microbial and endotoxins content during storage, this form of Water for Injection should be prepared and stored in a manner that limits microbial introduction and growth and/or should be used in a timely fashion before microbial proliferation renders it unsuitable for its intended use. Also, depending on the material used for packaging, extractable compounds could be leaching into the water from the packaging. Although this article is required to meet the same chemical purity standards as the bulk water, extractables from the packaging will likely render the packaged water less chemically pure than the bulk water. The nature of these impurities may even render the water an inappropriate choice for some applications. It is the user’s responsibility to ensure fitness for use of this packaged article when it is used in manufacturing, clinical, or analytical applications where the purer bulk form of the water is indicated.

### 3.1.3 WATER FOR HEMODIALYSIS

Water for Hemodialysis (see the USP monograph) is used for hemodialysis applications, primarily the dilution of hemodialysis concentrate solutions. The minimal quality of source water for the production of Water for Hemodialysis is Drinking Water whose attributes are prescribed by the U.S. EPA, EU, Japan, or WHO. Water for Hemodialysis has been further purified to reduce chemical and microbiological components, and it is produced and used on site. This water contains no added antimicrobial agents, and it is not intended for injection. Water for Hemodialysis must meet all of the chemical requirements specified in the monograph as well as an additional bacterial endotoxin specification. The microbial limits attribute for this water is unique among the “bulk” water monographs, but is justified on the basis of this water’s specific application, which has microbial content requirements related to its safe use. The bacterial endotoxins attribute is likewise established at a level related to its safe use.

### 3.1.4 PURE STEAM

Pure Steam (see the USP monograph) is also sometimes referred to as “clean steam”. It is used where the steam or its condensate would directly contact official articles or article-contact surfaces, such as during their preparation, sterilization, or cleaning where no subsequent processing step is used to remove any impurity residues. These Pure Steam applications include, but are not limited to, porous load sterilization processes, product or cleaning solutions heated by direct steam injection, or humidification of processes where steam injection is used to control the humidity inside processing vessels where the official articles or their in-process forms are exposed. The primary intent of using this quality of steam is to ensure that official articles or article-contact surfaces exposed to it are not contaminated by residues within the steam.

The minimal quality of source water for the production of Pure Steam is Drinking Water whose attributes are prescribed by the U.S. EPA, EU, Japan, or WHO, and which has been suitably treated. The water is then vaporized with suitable mist elimination, and distributed under pressure. The sources of undesirable contaminants within Pure Steam could arise from entrained source water droplets, anticorrosion steam additives, or residues from the steam production and distribution system itself. The chemical tests in the Pure Steam monograph should detect most of the contaminants that could arise from these sources. If an official article is exposed to Pure Steam and it is intended for parenteral use or other applications where the pyrogenic content must be controlled, the Pure Steam must additionally meet the specification for Bacterial Endotoxins Test (RS).

These purity attributes are measured in the condensate of the article, rather than the article itself. This, of course, imparts great importance to the cleanliness of the process for Pure Steam condensate generation and collection, because it must not adversely impact the quality of the resulting condensed fluid.

Other steam attributes not detailed in the monograph, particularly the presence of even small quantities of noncondensable gases or the existence of a superheated or dry state, may also be important for applications such as sterilization. The large release of energy (latent heat of condensation) as water changes from the gaseous to the liquid state is the key to steam’s sterilization efficacy and its efficiency, in general, as a heat transfer agent. If this phase change (condensation) is not allowed to happen because the steam is extremely hot and is in a persistent superheated, dry state, then its usefulness could be seriously compromised. Noncondensable gases in steam tend to stratify or collect in certain areas of a steam sterilization chamber or its load. These surfaces would thereby be at least partially insulated from the steam condensation phenomenon, preventing them from experiencing the full energy of the sterilizing conditions. Therefore, control of these kinds of steam attributes, in addition to its chemical purity, may also be important for certain Pure Steam applications. However, because these additional attributes are use-specific, they are not mentioned in the Pure Steam monograph.

Note that lower-purity “plant steam” may be used in the following applications: 1) for steam sterilization of nonproduct-contact nonporous loads, 2) for general cleaning of nonproduct-contact equipment, 3) as a nonproduct-contact heat-exchange medium, and 4) in all compatible applications involved in bulk pharmaceutical chemical and API manufacture.

Finally, because Pure Steam is lethal to microbes, monitoring of microbial control within a steam system is unnecessary, as is microbial analysis of the steam condensate.

### 3.2 Sterile Monographed Waters

The following monographed waters are packaged forms of either Purified Water or Water for Injection that have been sterilized to preserve their microbiological properties. These waters may have specific intended uses as indicated by their names, and may also have restrictions on the packaging configurations related to those uses. In general, these sterile waters may be used in a variety of applications in lieu of the bulk forms of water from which they were derived. However, there is a substantial difference between the acceptance criteria for the chemical purities of these bulk waters versus sterile waters. The specifications for sterile waters differ from those of bulk waters to accommodate a wide variety of packaging types, properties, volumes, and uses. As a result, the inorganic and organic impurity specifications are not equivalent for bulk and packaged waters. The packaging materials and elastomeric closures are the primary sources of these impurities, which tend to increase over the shelf life of these packaged articles. Therefore, due consideration must be given to the chemical purity suitability at the time of use of the sterile forms of water when used in manufacturing, analytical, and cleaning applications in lieu of the bulk waters from
which these waters were derived. It is the user’s responsibility to ensure fitness for use of these sterile packaged waters in these applications. Nevertheless, for the applications discussed below for each sterile water, their respective purities and packaging restrictions generally render them suitable by definition.

3.2.1 STERILE PURIFIED WATER

Sterile Purified Water (see the USP monograph) is Purified Water, packaged and rendered sterile. It can be used in the preparation of nonparenteral compendial dosage forms or in analytical applications requiring Purified Water where 1) access to a validated Purified Water system is not practical, 2) only a relatively small quantity is needed, 3) Sterile Purified Water is required by specific monograph or pharmacy practice, or 4) bulk packaged Purified Water is not suitably controlled for the microbiological quality for its intended use.

3.2.2 STERILE WATER FOR INJECTION

Sterile Water for Injection (see the USP monograph) is Water for Injection packaged and rendered sterile. It is used for extemporaneous prescription compounding and as a sterile diluent for parenteral products. It may also be used for other applications where bulk Water for Injection or Purified Water is indicated but access to a validated water system is not practical, or where only a relatively small quantity is needed. Sterile Water for Injection is packaged in single-dose containers not larger than 1 L.

3.2.3 BACTERIOSTATIC WATER FOR INJECTION

Bacteriostatic Water for Injection (see the USP monograph) is Water for Injection, packaged and rendered sterile, to which has been added one or more suitable antimicrobial preservatives. It is intended to be used as a diluent in the preparation of parenteral products, most typically for multi-dose products that require repeated content withdrawals. It may be packaged in single-dose or multiple-dose containers not larger than 30 mL.

3.2.4 STERILE WATER FOR IRRIGATION

Sterile Water for Irrigation (see the USP monograph) is Water for Injection packaged and sterilized in single-dose containers that may be larger than 1 L and allow rapid delivery of their contents. Due to its usage, Sterile Water for Irrigation is not required to meet Particulate Matter in Injections (788). It may also be used in other applications that do not have particulate matter specifications, where bulk Water for Injection or Purified Water is indicated but where access to a validated water system is not practical, or where somewhat larger quantities are needed than are provided as Sterile Water for Injection.

3.2.5 STERILE WATER FOR INHALATION

Sterile Water for Inhalation (see the USP monograph) is Water for Injection that is packaged and rendered sterile and is intended for use in inhalators and in the preparation of inhalation solutions. This monograph has no requirement to meet (788); it carries a less stringent specification for bacterial endotoxins than Sterile Water for Injection, and therefore is not suitable for parenteral applications.

3.3 Nonmonographed Waters

In addition to the bulk monographed waters described above, nonmonographed waters can also be used in pharmaceutical processing steps such as cleaning and synthetic steps, and also as a starting material for further purification or testing purposes. Unless otherwise specified in the compendium, the minimum quality of water is Purified Water. [NOTE—The information in this chapter is not an all-inclusive discussion of all nonmonographed waters identified in the USP–NF.]

3.3.1 DRINKING WATER

Drinking Water can be referred to as Potable Water (meaning drinkable or fit to drink), National Primary Drinking Water, Primary Drinking Water, or EPA Drinking Water. Except where a singular drinking water specification is stated (such as the U.S. EPA’s NPDWR, as cited in 40 CFR Part 141), this water must comply with the quality attributes of either the NPDWR or the drinking water regulations of the EU or Japan, or the WHO Guidelines for Drinking-Water Quality. Drinking Water may originate from a variety of sources including a public water supply, a private water supply (e.g., a well), or a combination of these sources (see 2. Source Water Considerations).

Drinking Water may be used in the early stages of cleaning pharmaceutical manufacturing equipment and product-contact components. Drinking Water is also the minimum quality of water that should be used for the preparation of official substances and other bulk pharmaceutical ingredients. Where compatible with the processes, the contaminant levels allowed in Drinking Water are generally considered safe for use in preparing official substances and other drug substances. However, where required by the processing of the materials to achieve their required final purity, higher qualities of water may be needed for these manufacturing steps, perhaps even water as pure as Water for Injection or Purified Water. Such higher-purity waters, however, might require only selected attributes to be of higher purity than Drinking Water (see Figure 2a and Figure 2b). Drinking Water is the prescribed source or feed water for the production of bulk monographed pharmaceutical waters. The use of Drinking Water specifications establishes a reasonable set of maximum allowable levels of chemical and microbiological contaminants with which a water purification system will be challenged. Because seasonal variations in the quality attributes of the Drinking Water supply can occur, it is important to give due consideration to its uses. The processing steps in the production of pharmaceutical waters must be designed to accommodate this variability.
In addition to Drinking Water, this compendium discusses waters with various other designations. These include waters of various quality levels for special uses such as, but not limited to, cleaning and testing purposes. Both General Notices and Requirements (see General Notices, 8.230.30 Water in a Compendial Procedure) and Reagents, Indicators, and Solutions clearly state that where the term “water” is indicated for use in analyses without grammatical qualification or other specification, the quality of the water must be Purified Water. However, numerous such qualifications do exist. Some of these qualifications involve adjectives describing methods of preparation, ranging from specifying the primary purification step to specifying additional purification. Other qualifications call for specific attribute “absences” to be met that might otherwise interfere with analytical processes. In most of these cases, the required attribute absences are not specifically tested. Sometimes, a further “purification process” is specified that ostensibly allows the water to adequately meet this required “absence attribute”.

However, preparation instructions for many reagents were carried forward from the innovator’s laboratories to the originally introduced monograph for a particular USP–NF article or general test chapter. The quality of the reagent water described in these tests may reflect the water quality designation of the innovator’s laboratory. These specific water designations may have originated without the innovator’s awareness of the requirement for Purified Water in USP–NF tests. Regardless of the original reason for the creation of these numerous special analytical waters, it is possible that the attributes of these special waters could now be met by the basic preparation steps and current specifications of Purified Water. In some cases, however, some of the cited post-processing steps are still necessary to reliably achieve the required attributes.

Users are not obligated to utilize specific and perhaps archaically generated forms of analytical water where alternatives with equal or better quality, availability, or analytical performance may exist. The consistency and reliability of operations for producing these alternative analytical waters should be verified so that the desired attributes are produced. In addition, any alternative analytical water must be evaluated on an application-by-application basis by the user to ensure its suitability. The following is a summary of the various types of nonmonographed analytical waters that are cited in the USP–NF. This is not an exhaustive listing. Those listed below are used in multiple locations. Several nonmonographed analytical waters are not included below because they are only found in one or perhaps two locations within this compendium.
Note that the names of many of the waters below imply a very low chemical impurity level. For example, “deionized water” implies that all the ions have been removed. However, in most cases discussed below, exposure of the water to air will result in the ingress of carbon dioxide (CO₂), leading to the formation of bicarbonate and hydrogen ions. Therefore, the removal of ions cannot be completely maintained for most analytical applications.

3.3.3 AMMONIA-FREE WATER

From a functional standpoint, Ammonia-Free Water must have a negligible ammonia concentration to avoid interference in tests sensitive for or to ammonia. Due to the nature of the uses of this water, Purified Water could be a reasonable alternative for these applications.

3.3.4 CARBON DIOXIDE-FREE WATER

Carbon dioxide-free water is defined in the Reagents, Indicators, and Solutions section of USP–NF as Purified Water that has been vigorously boiled for NLT 5 min, then cooled and protected from absorption of atmospheric carbon dioxide. Alternatively, this could be Purified Water that has a resistivity of NLT 18 megohm-cm at 25°C.

Because the absorption of atmospheric carbon dioxide lowers the pH of high-purity waters, most of the uses of Carbon Dioxide-Free Water are either associated as a solvent in pH-related or pH-sensitive determinations or as a solvent in bicarbonate-sensitive reagents or determinations.

The term “Carbon Dioxide-Free Water” is sometimes used improperly. Besides its use for pH or acidity/alkalinity tests, the purpose for using this water is not always clear. The intention could be to use water that was deaerated (free of dissolved air) or deionized (free of extraneous ions), or even Purified Water with an additional boiling step. Although boiling is highly effective for removing carbon dioxide as well as all other dissolved gasses, these gases are readily re-absorbed unless the water is protected. Even with protection, such as use of a stoppered container, re-absorption will occur over time as air will readily transmit through seals and diffuse through most materials. Deionization is also an efficient process for removing dissolved carbon dioxide. Carbon dioxide forms ionic bicarbonate in water, and will be subsequently removed by ion-exchange resins. However, the same problem of carbon dioxide re-absorption will occur after the deionized water is exposed to air. Also, the deionization approach for creating Carbon Dioxide-Free Water does not deaerate the water or remove other dissolved gases such as oxygen (O₂), it only removes carbon dioxide and other ions.

Depending on the application, Purified Water may meet the requirements where Carbon Dioxide-Free Water is called for. This could also include pH or acidity or alkalinity tests. The pH of a sample of pure Deionized Water is, by definition, 7.0. When that same sample is exposed to typical environmental atmospheric conditions, the water sample will absorb carbon dioxide and result in a pH range of approximately 5.4–6.2 ([H⁺] is in the range of 4.0 × 10⁻⁶ M to 6.3 × 10⁻⁷ M). The added acidity caused by carbon dioxide absorption may be insignificant compared to the material being analyzed.

3.3.5 DISTILLED WATER

Distilled Water is produced by vaporizing Drinking Water or a higher quality of water and condensing it into a purer state. It is used primarily as a solvent for reagent preparation, and it is also specified in the execution of other aspects of tests, such as for rinsing an analyte, transferring a test material as a slurry, as a calibration standard or analytical blank, and for test apparatus cleaning. Distilled Water is also cited as the starting water to be used for making High-Purity Water (see 3.3.10 High-Purity Water). Because none of the cited uses of this water imply a need for a particular purity attribute that can only be derived by distillation, water meeting the requirements for Purified Water derived by other means of purification or Water for Injection could be equally suitable where Distilled Water is specified. It is the user’s responsibility to verify the suitability of Purified Water or Water for Injection.

3.3.6 FRESHLY DISTILLED WATER

Freshly Distilled Water or “recently distilled water” is produced in the same manner as Distilled Water and should be used soon after its generation. This implies that all the ions have been removed. However, in most cases discussed below, exposure of the water to air will result in the ingress of carbon dioxide (CO₂), leading to the formation of bicarbonate and hydrogen ions. Therefore, the removal of ions cannot be completely maintained for most analytical applications.

3.3.7 DEIONIZED WATER

Deionized Water can be produced by starting with either Drinking Water or Purified Water, depending upon monograph or testing procedures defined in the compendia. Deionized Water is produced by an ion-exchange process in which the cations and anions are replaced with H⁺ and OH⁻ ions by use of ion-exchange resins. Similar to Distilled Water, Deionized Water is used primarily as a solvent for reagent preparation, but it is also specified in the execution of other aspects of tests, such as for transferring an analyte within a test procedure, as a calibration standard or analytical blank, and for test apparatus cleaning. Also, none of the cited uses of this water imply any needed purity attribute that can only be achieved by deionization. Therefore, water meeting the requirements for Purified Water that is derived by other means of purification could be equally suitable where Deionized Water is specified. It is the user’s responsibility to verify the suitability of Purified Water.
3.3.8 DEIONIZED DISTILLED WATER

Deionized Distilled Water is produced by deionizing (see 3.3.7 Deionized Water) Distilled Water. This water is used as a reagent in a liquid chromatography test that requires a low ionic or organic impurity level. Because of the importance of this high purity, water that meets the requirements for Purified Water may not be acceptable. High-Purity Water (see 3.3.10 High-Purity Water) could be a reasonable alternative to this water. It is the user’s responsibility to verify the suitability of the alternative water used.

3.3.9 FILTERED WATER

Filtered Water is Purified Water that has been filtered to remove particles that could interfere with the analysis where this water is specified. It is sometimes used synonymously with Particle-Free Water and Ultra-Filtered Water and is cited in some monographs and general chapters as well as in Reagents, Indicators, and Solutions. Depending on its referenced location in USP–NF, it is variously defined as water that has been passed through filters rated as 1.2, 0.2, or 0.22 µm, or unspecified porosity rating. Even though the water names and the filter ratings used to produce these waters are defined inconsistently, the use of 0.2-µm or 0.22-µm filtered Purified Water should be universally acceptable for all applications where Particle-Free Water, Filtered Water, or Ultra-Filtered Water are specified.

3.3.10 HIGH-PURITY WATER

High-Purity Water may be prepared by deionizing previously distilled water and then filtering it through a 0.45-µm rated membrane. This water must have an in-line conductivity of NMT 0.15 µS/cm (NLT 6.67 megohm-cm) at 25°C. If the water of this purity contacts the atmosphere even briefly as it is being used or drawn from its purification system, its conductivity will immediately increase by as much as about 1.0 µS/cm * as atmospheric carbon dioxide dissolves in the water and equilibrates to hydrogen and bicarbonate ions. Therefore, if the analytical use requires that water conductivity remains as low as possible or the bicarbonate/carbon dioxide levels be as low as possible, the water should be protected from atmospheric exposure. High-Purity Water is used as a reagent, as a solvent for reagent preparation, and for test apparatus cleaning where less stringent water specifications would not be considered acceptable. However, if a user’s routinely available Purified Water is filtered and meets or exceeds the conductivity specifications of High-Purity Water, it could be used in lieu of High-Purity Water.

3.3.11 DEAERATED WATER

Deaerated Water or “degassed water” is Purified Water that has been treated to reduce the content of dissolved air by “suitable means” such as boiling, sonication, and/or stirring during the application of a partial vacuum, followed by immediate use or protection from air reabsorption.

3.3.12 OXYGEN-FREE WATER

Oxygen-Free Water is Purified Water that has been treated to remove or reduce dissolved oxygen. Such treatment could involve deaerating by boiling or sparging with an inert gas such as nitrogen or helium, followed by inert gas blanketing to prevent oxygen reabsorption. Any procedure used for removing oxygen should be verified as reliably producing water that is fit for use.

3.3.13 WATER FOR BACTERIAL ENDOXINS TEST

Water for Bacterial Endotoxins Test (BET) is also referred to as Limulus Amebocyte Lysate (LAL) Reagent Water. This type of water is often Water for Injection, which may have been sterilized. It is free from a level of endotoxin that would yield any detectable reaction or interference with the LAL reagent used in the BET (see 85).

Change to read:

4. VALIDATION AND QUALIFICATION OF WATER PURIFICATION, STORAGE, AND DISTRIBUTION SYSTEMS

4.1 Validation Requirement

Establishing the reliability of pharmaceutical water purification, storage, and distribution systems requires demonstrating control of the process through an appropriate period of monitoring and observation. Finished water is typically continuously produced and used, while product and process attributes may only be periodically assessed. The quality of bulk finished water cannot be established by only testing monograph attributes. The unit operations in the pharmaceutical water system need to demonstrate that they are in control through monitoring of the process parameters and water quality. The advent of using conductivity and total organic carbon (TOC) to define chemical purity allows the user to more quantitatively assess the water’s chemical purity and its variability as a function of routine treatment system maintenance and regeneration. Treatment processes must also demonstrate control of microbial attributes within the overall system. Some unit operations that are needed for chemical treatment may significantly increase microbial and bacterial endotoxin levels. These are later controlled by downstream unit operations. Knowledge of the treatment system processes and the effectiveness of control measures is needed to ensure that the pharmaceutical waters are acceptable for use.

Efficacy of the design, operation, sanitization, and control of the pharmaceutical water system is demonstrated through the monitoring of chemical and microbial attributes. A typical water system validation program involves an initial increased
frequency of monitoring of the treatment system process parameters and sampling and testing of major process points to demonstrate the ability to produce the acceptable water and to characterize the operation of the system. This is followed by a life cycle approach of validation maintenance and monitoring.

4.2 Validation Approach

Validation is the program of documenting, to a high level of assurance, that a specific process is capable of consistently delivering product conforming to an established set of quality attributes. A validation program qualifies and documents the design, installation, operation, and performance of the system. A graphical representation of a typical water system validation life cycle is shown in Figure 3.

The validation protocol should be based on the boundaries of the water system and the critical water quality and process attributes needed to maintain consistent performance. The system boundary may stop at the point of use or may include the water transfer process. If the transfer process from the distribution system outlets to the water use locations (typically either with hoses or hard-piped equipment connections) is defined as outside the water system boundary, then this transfer process still needs to be validated to not adversely affect the quality of the water as it is delivered for use. Because routine quality control (QC) microbial monitoring is performed for the same transfer process and components (e.g., hoses and heat exchangers) as that of routine water use (see 6.1.2 QC Sampling), there is some logic to include this water transfer process within the distribution system validation.

4.2.1 VALIDATION ELEMENTS

Validation is accomplished through the use of a structured, documented process. The phases of this process include Design Qualification (DQ), Installation Qualification (IQ), Operational Qualification (OQ), Performance Qualification (PQ), and Validation Maintenance. The process is documented in a validation protocol. The elements may be in individual protocols for each phase, or integrated into variations of a DQ/IQ/OQ/PQ combined document format. The protocols are formally approved quality documents. Factory Acceptance Testing (FAT), Site Acceptance Testing (SAT), and commissioning testing of the system may supplement qualification tests for IQ or OQ provided that they are properly documented and reviewed; and if it can be shown that the system functionality is not affected by the transport and installation.

![Figure 3. Water system validation life cycle.](not official text. Please refer to the currently official version of the applicable USP–NF or FCC standard for compliance purposes.)

### 4.2.2 USER REQUIREMENTS SPECIFICATION AND DESIGN QUALIFICATION

The user requirements for the water system should identify the design, operation, maintenance, and quality elements needed to produce the desired water type from the available source water, including its anticipated attribute variability. The essential elements of quality need to be built in at this stage and any GMP risks mitigated to an acceptable level.

The review of the specifications, system design, components, functions, and operation should be performed to demonstrate that the system complies with GMPs and verify that the design meets the user requirements. This documented review may be performed as part of the overall design process or as a separate DQ.
4.2.3 IQ

An IQ protocol for a water system confirms that the system has been properly installed and documented. This may include verification of components, piping, installation, and weld quality; documentation of the specifications for all system components present; inspections to verify that the drawings accurately depict the final configuration of the water system and, where necessary, special tests to verify that the installation meets the design requirements. Additionally, the water system is readied for operational testing, including calibration of instruments, configuration of alarm levels and adjustment of operating parameters (e.g., flow rate, pressure).

4.2.4 OQ

The OQ phase consisting of tests and inspections to verify that the equipment, system alerts, and controls are operating reliably and that appropriate Alert and Action Levels are established (this phase of qualification may overlap with aspects of IQ and PQ). During this phase of validation specific testing is performed for alarms, verifying control sequences, equipment functional checks, and verification of operating ranges. SOPs for all aspects of water system operation, maintenance, water use, water sampling, and testing, etc. should be in place and operator training completed. At the completion of the OQ, the water system has demonstrated that the components are operational and the system is producing suitable water.

4.2.5 PQ

The prospective PQ stage considers two aspects of the water system: critical process parameters and critical water attribute parameters. These are evaluated in parallel by monitoring the water quality and demonstrating acceptable quality attributes while demonstrating control of the process parameters (see 6.3 Validation Sampling Plans). The initial PQ stage may result in refinement of process parameters to yield appropriate water quality. This PQ stage includes an increased frequency of monitoring for approximately 2–4 weeks, or sufficient time to generate adequate data to demonstrate that water meeting the appropriate quality attributes is produced and distributed. One of the reasons for this duration is that biofilm, the source of planktonic organisms in water samples, takes time to develop and to determine if the sanitization unit operations and processes are adequate to control microbial proliferation. The chemical control program adequacy is typically apparent in less time than it takes to see microbial control adequacy. However, chemical purification can be compromised by poor microbial control and, to a lesser degree, vice versa.

Once a level of control of microbial and chemical attributes has been demonstrated, the next phase of PQ is to continue the frequency of monitoring for approximately 2–4 weeks at a somewhat reduced level that will still give adequate data on system performance while using the pharmaceutical water. The water may be used for manufacturing at risk, and the associated products may be released only after water quality attributes have been determined to be acceptable and this validation phase has been completed. At the completion of the second phase, the data should be formally reviewed and the system approved for operational use.

4.3 Operational Use

When the water system has been placed into operational use, monitoring of the water quality attributes and the system process parameters is performed at a routine frequency (see 6.4 Routine Sampling Plans) to ensure that they remain with a state of control during long-term variability from seasonal variations in source water quality, unit operation maintenance, system sanitization processes, and earlier-established Alert and Action Levels.

The water system should continue to be monitored and evaluated on an on-going basis following a life cycle approach using online instruments or samples for laboratory-based testing. The use of online instruments and process automation technology, such as conductivity, TOC, temperature, flow rate, and pressure can facilitate improved operational control of the attributes and parameters and for process release. Manual observation of operating parameters and laboratory-based testing is also appropriate and acceptable for monitoring and trend evaluation.

4.3.1 MONITORING

The frequency of routine monitoring should be based on the criticality of the finished water, capabilities of the process, and ability to maintain product water quality trends. Monitoring may be adjusted from the initial validation monitoring program when there is sufficient data to support a change (see 6.4 Routine Sampling Plans).

4.3.2 VALIDATION MAINTENANCE

Maintaining the validated state of control requires a life cycle approach. After the completion of the PQ and release of the water system for use, ongoing activities and programs have to be in place to maintain the validated state of control after the system has been validated and placed into service (see 5.4 Operation, Maintenance, and Control). This includes unit operation, calibration, corrective maintenance, preventive maintenance, procedures, manuals and drawings, standardization of instruments, process parameter and quality attribute trending, change control, deviations, corrective and preventive actions (CAPA), training, records retention, logbooks, etc.

4.3.3 CHANGE CONTROL

Identification and control of changes made to unit operations and other system components, operation parameters, system sanitization, and laboratory processes or procedures need to be established. Not all changes will require validation follow up, but even minor ones, such as gasket elastomer changes could have an impact on quality attributes. The impact of the change
on process parameters and quality attributes must be identified, evaluated and remediated. This may result in a selective validation activity to demonstrate the ongoing state of control for the system and ability to maintain water quality attributes.

Certain calibration and preventive maintenance activities may be considered routine tasks if they do not impact on system operation or water quality. Replacement of components needs to be carefully evaluated. Replacement of components using exact parts generally does not affect system operation or control. Replacement of components with ones that are not exact parts but have similar functional specifications can be performed at risk with the critical specifications (e.g., material of construction, dimensions, flow rate, response factors) having been evaluated and the differences determined to be acceptable and documented within the change control system.

### 4.3.4 PERIODIC REVIEW

The water system qualification, maintenance history, calibration records, quality and process data, issues with the unit operations and any process variability, change control, and other validation maintenance data should be assessed periodically to determine the state of control.

The review may result in adjustments to operating or sanitization processes, calibration or maintenance plans, or monitoring plans. This may also result in additional testing or repeating certain qualification tasks (re-qualification).

**Change to read:**

### 5. DESIGN AND OPERATION OF PURIFIED WATER AND WATER FOR INJECTION SYSTEMS

The design, installation, and operation of systems to produce Purified Water and Water for Injection include similar components, control techniques, and procedures. The quality attributes of the two waters differ in their bioburden expectation, the presence of a bacterial endotoxin requirement for Water for Injection, and in their methods of preparation. The similarities in the quality attributes provide considerable common ground in the design of water systems to meet either requirement. The critical difference is the degree of control of the system and the final purification steps needed to ensure removal of bacteria and bacterial endotoxins and reductions in opportunities for biofilm re-development within those purification steps that could become in situ sources of bacteria and endotoxin in the finished water.

Many aspects of system design and operation relate to control and elimination of biofilm. Unit operations can cause the deterioration of water microbial attributes and the formation of biofilm on unit operation surfaces, even when properly maintained (see 8.2 Biofilm Formation in Water Systems).

Production of pharmaceutical water involves sequential unit operations (processing steps) that address specific water quality attributes and protect the operation of subsequent treatment steps. A typical evaluation process for selecting an appropriate purification system needs to take into consideration different aspects, including the source water quality, sanitization, operation used to produce Water for Injection is limited to distillation or other processes equivalent or superior to distillation in the removal of chemical impurities as well as microorganisms and their components, such as bacterial endotoxins. Distillation coupled with suitable pretreatment technologies has a long history of generally reliable performance (though not completely infallible) and can be validated as a unit operation for the production of Water for Injection. Other combinations of purification technologies may also be suitable in the production of Water for Injection if they can be shown through validation to be as effective and reliable as distillation in the removal of chemicals and microorganisms. The development of new designs and materials of construction for other technologies (such as reverse osmosis, electrodeionization, and ultrafiltration) that allow intermittent or continuous operation at hot bactericidal conditions show promise for a valid use in producing Water for Injection.

#### 5.1 Unit Operations Considerations

To achieve the quality attributes for pharmaceutical waters, multiple-unit operations are required. The design of the water purification system needs to take into consideration different aspects, including the source water quality, sanitization, pharmaceutical water quality attributes, uses of the water, and maintenance programs. Each unit operation contributes specific purification attributes associated with chemical and microbiological parameters.

The following is a brief description of selected unit operations and the design, installation, operation, maintenance, and monitoring parameter considerations associated with them. Not all unit operations are discussed, nor are all potential shortcomings addressed.

##### 5.1.1 PREFILTRATION

The purpose of prefiltration—also referred to as initial, coarse, particulate, or depth filtration—is to remove solid contaminants from the incoming source water supply and protect downstream system components from particulates that can inhibit equipment performance and shorten their effective life. This coarse filtration technology primarily uses sieving effects for particle capture and a depth of filtration medium that has a high “dirt load” capacity. Such filtration units are available in a wide range of designs and for various applications. Removal efficiencies and capacities differ significantly, from granular bed filters such as multimedia or sand for larger water systems, to depth cartridges for smaller water systems. Unit and system configurations vary widely in the type of filtering media and the location in the process. Granular or cartridge prefilters are often situated at the beginning of the water purification system prior to unit operations designed to remove the source water disinfectants.

Cartridge-type coarse filters may also be used to capture fines released from granular beds such as activated carbon and deionization beds. These locations, however, do not preclude the need for periodic microbial evaluation.

Design and operational issues that may impact the performance of depth filters include channeling of the filtering media, blockage from silt, microbial growth, and filtering-media loss during improper backwashing. Control methods involve pressure
and flow monitoring during use and backwashing, sanitizing, and replacing filtering media. An important design concern is sizing of the filter to prevent channeling or media loss resulting from inappropriate water flow rates as well as proper sizing to minimize excessively frequent or infrequent backwashing or cartridge filter replacement.

5.1.2 ACTIVATED CARBON

Activated carbon beds, depending on the type and placement, are used to adsorb low-molecular-weight organic material, bacterial endotoxins, and oxidizing additives such as chlorine and chloramine compounds, removing them from the water. They are used to achieve certain quality attributes and to protect against reactions with downstream unit operations, stainless steel surfaces, resins, and membranes.

The chief operating concerns regarding activated carbon beds include the propensity to support bacterial growth, the potential for hydraulic channeling, the organic adsorption capacity, and insufficient contact time. Operation deficiencies may result in the release of bacteria, endotoxins, organic chemicals, and fine carbon particles.

Control measures may involve monitoring water flow rates and differential pressures, sanitizing with hot water or steam, backwashing, testing for adsorption capacity, and frequent replacement of the carbon bed. Monitoring of carbon bed unit operation may also include microbial loading, disinfectant chemical reduction, and TOC if used for TOC reduction. The use of hot water or steam for carbon bed sanitization is ineffective if there is channeling rather than even permeation through the bed. Channeling can be mitigated through design and proper flow rates during sanitization.

Microbial biofilm development on the surface of the granular carbon particles can cause adjacent bed granules to agglomerate. This may result in ineffective removal of trapped debris and fragile biofilm during backwashing, and ineffective sanitization.

Alternative technologies to activated carbon beds can be used to avoid their microbial challenges. These include disinfectant-neutralizing chemical additives and intense ultraviolet (UV) light for removal of chlorine, and regenerable organic scavenging deionizing resins for removal of organics.

5.1.3 ADDITIVES

Chemical additives are used in water systems 1) to control microorganisms by use of sanitizing agents, such as chlorine compounds and ozone; 2) to enhance the removal of suspended solids by use of flocculating agents; 3) to remove chlorine compounds; 4) to avoid scaling on reverse osmosis membranes; and 5) to adjust pH for more effective removal of carbonate and ammonia compounds by reverse osmosis. These additives do not constitute “added substances” as long as they are either removed by subsequent processing steps or are otherwise absent from the finished water. Control of additives to ensure a continuously effective concentration and subsequent monitoring to ensure their removal should be designed into the system and included in the monitoring program.

5.1.4 ORGANIC SCAVENGERS

Organic scavenging devices use macroreticular, weakly basic anion-exchange resins capable of removing negatively charged organic material and endotoxins from the water. Organic scavenger resins can be regenerated with appropriate biocidal caustic brine solutions. Operating concerns are associated with organic scavenging capacity; particulate, chemical, and microbiological fouling of the reactive resin surface; flow rate; regeneration frequency; and shedding of fines from the fragile resins. Control measures include TOC testing of influent and effluent, backwashing, monitoring hydraulic performance, and using downstream filters to remove resin fines.

5.1.5 SOFTENERS

Water softeners may be located either upstream or downstream of disinfectant removal units. They utilize sodium-based cation-exchange resins to remove water-hardness ions, such as calcium and magnesium, that could foul or interfere with the performance of downstream processing equipment such as reverse osmosis membranes, deionization devices, and distillation units. Water softeners can also be used to remove other lower affinity cations, such as the ammonium ion, that may be released from chloramine disinfectants commonly used in drinking water. If ammonium removal is one of its purposes, the softener must be located downstream of the disinfectant removal operation. Water softener resin beds are regenerated with concentrated sodium chloride solution (brine).

Concerns include microorganism proliferation, channeling, appropriate water flow rates and contact time, ion-exchange capacity, organic and particulate resin fouling, organic leaching from new resins, fracture of the resin beads, resin degradation by excessively chlorinated water, and contamination from the brine solution used for regeneration.

Control measures involve recirculation of water during periods of low water use; periodic sanitization of the resin and brine system; use of microbial control devices (e.g., UV light and chlorine); locating the unit upstream of the disinfectant removal step (if used only for softening); appropriate regeneration frequency; effluent chemical monitoring (e.g., hardness ions and possibly ammonium); and downstream filtration to remove resin fines. If a softener is used for ammonium removal from chloramine-containing source water, then the capacity, contact time, resin surface fouling, pH, and regeneration frequency are very important.

5.1.6 DEIONIZATION

Deionization (DI) and continuous electrodeionization (CEDI) are effective methods of improving the chemical quality attributes of water by removing cations and anions. DI systems have charged resins that require periodic regeneration with an acid and base. Typically, cation resins are regenerated with either hydrochloric or sulfuric acid, which replace the captured positive ions with hydrogen ions. Anion resins are regenerated with sodium hydroxide or potassium hydroxide, which replace captured negative ions with hydroxide ions. Because free endotoxin is negatively charged, some removal of endotoxin is
achieved by the anion resin. The system can be designed so that the cation and anion resins are in separate or “twin” beds, or they can be blended together to form a “mixed” bed.

The CEDI system uses a combination of ion-exchange materials such as resins or grafted material, selectively permeable membranes, and an electric charge, providing continuous flow (of product and waste concentrate) and continuous regeneration. Water enters both the resin section and the waste (concentrate) section. The resin acts as a conductor, enabling the electrical potential to drive the captured cations and anions through the resin and appropriate membranes for concentration and removal in the waste water stream. As the water passes through the resin, it is deionized to become product water. The electrical potential also separates the water in the resin (product) section into hydrogen and hydroxide ions. This permits continuous regeneration of the resin without the need for regenerant additives. However, unlike conventional deionization, CEDI units must start with water that is already partially purified because they generally cannot achieve the conductivity attribute of Purified Water when starting with the heavier ion load of source water.

Concerns for all forms of DI units include microbial and endotoxin control; chemical additive impact on resins and membranes; and loss, degradation, and fouling of resin. Issues of concern specific to DI units include regeneration frequency and completeness; channeling caused by biofilm agglomeration of resin particles; organic leaching from new resins; complete resin separation for mixed bed regeneration; and bed fluidization air contamination (mixed beds).

Control measures may include continuous recirculation loops, effluent microbial control by UV light, conductivity monitoring, resin testing, microporous filtration of bed fluidization air, microbial monitoring, frequent regeneration to minimize and control microorganism growth, sizing the equipment for suitable water flow and contact time, and use of elevated temperatures. Internal distributor and regeneration piping for DI bed units should be configured to ensure that regeneration chemicals contact all internal bed and piping surfaces and resins.

Rechargeable canisters can be the source of contamination and should be carefully monitored. Full knowledge of previous resin use, minimum storage time between regeneration and use, and appropriate sanitizing procedures are critical factors for ensuring proper performance.

5.1.7 REVERSE OSMOSIS

Reverse osmosis (RO) units use semipermeable membranes. The “pores” of RO membranes are intersegmental spaces among the polymer molecules. They are big enough for permeation of water molecules, but they limit the passage of hydrated chemical ions, organic compounds, and microorganisms. RO membranes can achieve chemical, microbial, and endotoxin quality improvement. Many factors, including pH, temperature, source water hardness, permeate and reject flow rate, and differential pressure across the membrane, affect the selectivity and effectiveness of this permeation. The process streams consist of supply water, product water (permeate), and waste water (reject). Depending on the source water, pretreatment and system configuration variations and chemical additives may be necessary to achieve the desired performance and reliability. For most source waters, a single stage of RO filtration is usually not enough to meet Purified Water conductivity specifications. A second pass of this permeate water through another RO stage usually achieves the necessary permeate purity if other factors such as pH and temperature have been appropriately adjusted and the ammonia from source water that has been previously treated with chloramines is removed.

Concerns associated with the design and operation of RO units include membrane materials that are sensitive to sanitizing agents and to particulate, chemical, and microbial membrane fouling; membrane and seal integrity; and the passage of dissolved gases, such as carbon dioxide and ammonia. Failure of membrane or seal integrity will result in product water contamination. Methods of control involve suitable pretreatment of the influent water stream; appropriate membrane material selection; membrane design and heat tolerance; periodic sanitization; and monitoring of differential pressures, conductivity, microbial levels, and TOC.

The development of RO units that can tolerate sanitizing water temperatures and also operate efficiently and continuously at elevated temperatures has added greatly to their microbial control ability and to the avoidance of biofouling. RO units can be used alone or in combination with DI and CEDI units, as well as ultrafiltration, for operational and quality enhancements.

5.1.8 ULTRAFILTRATION

Ultrafiltration is a technology that is often used near the end of a pharmaceutical water purification system for removing endotoxins from a water stream though upstream uses are possible. Ultrafiltration can use semipermeable membranes, but unlike RO, these typically use polysulphone membranes with intersegmental “pores” that have been purposefully enlarged. Membranes with differing molecular weight “cutoffs” can be created to preferentially reject molecules with molecular weights above these ratings.

Ceramic ultrafilters are another molecular sieving technology. Ceramic ultrafilters are self-supporting and extremely durable; they can be backwashed, chemically cleaned, and steam sterilized. However, they may require higher operating pressures than do membrane-type ultrafilters.

All ultrafiltration devices work primarily by a molecular sieving principle. Ultrafilters with molecular weight cutoff ratings in the range of 10,000–20,000 Da are typically used in water systems for removing endotoxins. This technology may be appropriate as an intermediate or final purification step. As with RO, successful performance is dependent upon pretreatment of the water by upstream unit operations.

Issues of concern for ultrafilters include compatibility of membrane material with heat and sanitizing agents, membrane integrity, fouling by particles and microorganisms, and seal integrity. Control measures involve filter membrane composition, sanitization, flow design (dead end vs. tangential), cartridge replacement, elevated feed water temperature, and monitoring TOC and differential pressure.
5.1.9 MICROBIAL-RETENTIVE FILTRATION

Microbial-retentive membrane filters have a larger effective “pore size” than ultrafilters and are intended to prevent the passage of microorganisms and similarly sized particles without unduly restricting flow. This type of filtration is widely employed within water systems for filtering the bacteria out of both water and compressed gases as well as for vent filters on tanks and stills and other unit operations.

In water systems, a filter’s microbial retention characteristics exhibit different phenomena than in other aseptic filtration applications.

The following factors interact to create the retention phenomena for water system microorganisms: the variability in the range and average pore sizes created by the various membrane fabrication processes; the variability of the surface chemistry and three-dimensional structure related to the different polymers used in these filter matrices; and the size and surface properties of the microorganism intended to be retained by the filters. ¹⁵ (USP41) In some situations, the appearance of water system microorganisms on the downstream sides of some 0.2- to 0.22-µm rated filters after a ¹⁵ (USP41) period of use (days to weeks) seems to support the idea that water-borne microorganisms can penetrate the 0.2- to 0.22-µm rated filters. ¹⁵ (USP41) It is not known whether this downstream appearance is caused by exceeding the retentive capabilities of the filters due to high pretreatment bioburden levels of water-borne microorganisms and extended filtration times. These conditions can lead to a “pass-through” phenomenon resulting from tiny cells or less cell “stickiness”, or perhaps by a “grow-through” phenomenon in which cells hypothetically replicate their way through the pores to the downstream side. ¹⁵ (USP41) Whatever the penetration mechanism, 0.2- to 0.22-µm rated membranes may not be the best choice for some water system uses (see Sterility Assurance (1211)). ¹¹ (CN 1-May-2018)

Nevertheless, microbial retention success in water systems has been reported with the use of filters rated as 0.2 or 0.1 µm. There is general agreement that, for a given manufacturer, their 0.1-µm rated filters are tighter than their 0.2- to 0.22-µm rated filters. However, comparably rated filters from different manufacturers may not have equivalent performance in water filtration applications because of the different filter materials, different fabrication processes, and nonstandardized microbial retention challenge processes currently used for defining the 0.1-µm filter rating. It should be noted that filters with a 0.1-µm rating may result in a lower flow rate compared to 0.2- to 0.22-µm filters, so whatever filters are chosen for a water system application, the user must verify that they are suitable for their intended application, use period, and use process, including flow rate.

For microbial retentive gas filtrations, the same sieving and adsorptive retention phenomena are at work as in liquid filtration, but the adsorptive phenomenon is enhanced by additional electrostatic interactions between the particles and filter matrix. These electrostatic interactions are so strong, particle retention for a given filter rating is significantly more efficient in gas filtration than in water or product-solution filtrations. These additional adsorptive interactions render filters rated at 0.2–0.22 µm unquestionably suitable for microbial retentive gas filtrations. When microbial retentive filters are used in these applications, the membrane surface is typically hydrophobic (non-wettable by water). A significant area of concern for gas filtration is blockage of tank vents by condensed water vapor, which can cause mechanical damage to the tank. Control measures include electrical or steam tracing and a self-draining orientation of vent filter housings to prevent accumulation of vapor condensate.

However, a continuously high filter temperature will take an oxidative toll on polypropylene components of the filter, so sterilization of the unit prior to initial use, and periodically thereafter, as well as regular visual inspections, integrity tests, and filter cartridge changes are recommended control methods.

In water applications, microbial retentive filters may be used downstream of unit operations that tend to release microorganisms or upstream of unit operations that are sensitive to microorganisms. Microbial retentive filters may also be used to filter water feeding the distribution system. It should be noted that regulatory authorities allow the use of microbial retentive filters within distribution systems or even at use points if they have been properly validated and are appropriately maintained. A point-of-use filter should only be intended to “polish” the microbial quality of an otherwise well-maintained system and not to serve as the primary microbial control device. The efficacy of system microbial control measures can only be assessed by sampling at all points of entry and at sampling points within the distribution system. As an added measure of protection, in-line UV lamps, appropriately sized for the flow rate (see 5.3 Sanitization), may be used just upstream of microbial retentive filters to inactivate microorganisms prior to their capture by the filter. This tandem approach tends to greatly delay potential microbial penetration phenomena and can substantially extend filter service life.

5.1.10 ULTRAVIOLET LIGHT

The use of low-pressure UV lights that emit a 254-nm wavelength for microbial control is discussed in 5.3 Sanitization, but the application of UV light in chemical purification is also emerging. This 254-nm wavelength is also useful in the destruction of ozone. At wavelengths around 185 nm (as well as at 254 nm), medium-pressure UV lights have demonstrated utility in the destruction of the chlorine-containing disinfectants used in source water as well as for interim stages of water pretreatment. High intensities of ¹⁸⁵ nm alone or ²⁵⁴ nm ¹⁵ (USP41) in combination with other oxidizing sanitants, such as hydrogen peroxide, have been used to lower TOC levels in recirculating distribution systems. The organics are typically converted to carbon dioxide, which equilibrates to bicarbonate, and incompletely oxidized carboxylic acids, both of which can easily be removed by polishing ion-exchange resins.

Areas of concern include inadequate UV intensity and residence time, gradual loss of UV emissivity with bulb age, gradual formation of a UV-absorbing film at the water contact surface, incomplete photodegradation during unforeseen source water hyperchlorination, release of ammonia from chloramine photodegradation, unapparent UV bulb failure, and conductivity degradation in distribution systems using 185-nm UV lights.

Control measures include regular inspection or emissivity alarms to detect bulb failures or film occlusions, regular UV bulb cleaning and wiping, downstream chlorine dosimeters (when used for dechlorination), downstream polishing deionizers (when used for TOC reduction), and regular (approximately yearly) bulb replacement. UV lamps generate heat during operation, which can cause failure of the lamps or increase the temperature of the water. Precautions should be in place to ensure that water flow is present to control excessive temperature increase.

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5.1.11 DISTILLATION

Distillation units provide chemical and microbial purification via thermal vaporization, mist elimination, and water vapor condensation. A variety of designs is available, including single effect, multiple effect, and vapor compression. The latter two configurations are normally used in larger systems because of their generating capacity and efficiency. Source water controls must provide for the removal of hardness and silica impurities that may foul or corrode the heat transfer surfaces, as well as the removal of those impurities that could volatize and condense along with the water vapor. In spite of general perceptions, even the best distillation process does not ensure absolute removal of contaminating ions, organics, and endotoxins. Most stills are recognized as being able to accomplish at least a 3–4 log reduction in these impurity concentrations. They are highly effective in sterilizing the feed water.

Areas of concern include carryover of volatile organic impurities such as trihalomethanes (see 2. Source Water Considerations) and gaseous impurities such as ammonia and carbon dioxide, faulty mist elimination, evaporator flooding, inadequate blow down, stagnant water in condensers and evaporators, pump and compressor seal design, pinhole evaporator and condenser leaks, and conductivity (quality) variations during start-up and operation.

Methods of control may involve the following: preliminary steps to remove both dissolved carbon dioxide and other volatile or noncondensable impurities; reliable mist elimination to minimize feed water droplet entrainment; visual or automated high-water-level indication to detect boiler flooding and boil over; use of sanitary pumps and compressors to minimize microbial and lubricant contamination of feed water and condensate; proper drainage during inactive periods to minimize microbial growth and accumulation of associated endotoxin in boiler water; blow down control to limit the impurity concentration effect in the boiler to manageable levels; on-line conductivity sensing with automated diversion to waste to prevent unacceptable water upon still start-up or still malfunction from getting into the finished water distribution system; and periodic testing for pinhole leaks to routinely ensure that condensate is not compromised by nonvolatized source water contaminants.

5.1.12 STORAGE TANKS

Storage tanks are included in water distribution systems to optimize processing equipment capacity. Storage also allows for routine maintenance within the purification system while maintaining continuous supply to meet manufacturing needs. Design and operation considerations are needed to prevent or minimize the development of biofilm, to minimize corrosion, to aid in the use of chemical sanitization of the tanks, and to safeguard mechanical integrity.

Areas of concern include microbial growth or corrosion due to irregular or incomplete sanitization and microbial contamination from un alarmed rupture disk failures caused by condensate-occluded vent filters.

Control considerations may include using closed tanks with smooth interiors, the ability to spray the tank headspace using spray balls on recirculating loop returns, and the use of heated, jacketed/insulated tanks. This minimizes corrosion and biofilm development and aids in thermal or chemical sanitization. Storage tanks require venting to compensate for the dynamics of changing water levels. This can be accomplished with a properly oriented and heat-traced filter housing fitted with a hydrophobic microbial retentive membrane filter affixed to an atmospheric vent. Alternatively, an automatic membrane-filtered compressed gas blanketing system may be used. In both cases, rupture disks equipped with a rupture alarm device should be used as a further safeguard for the mechanical integrity of the tank.

5.1.13 DISTRIBUTION SYSTEMS

Distribution system configuration should allow for the continuous flow of water in the piping by means of recirculation. Use of no recirculating, dead-end, or one-way systems or system segments should be avoided whenever possible. If not possible, these systems should be flushed periodically and monitored more closely. Experience has shown that continuously recirculated systems are easier to maintain. Pumps should be designed to deliver fully turbulent flow conditions to facilitate thorough heat distribution (for hot-water sanitized systems) as well as thorough chemical sanitant distribution. Turbulent flow also appears to either retard the development of biofilms or reduce the tendency of those biofilms to shed bacteria into the water. If redundant components, such as pumps or filters, are used, they should be configured and used to avoid microbial contamination of the system.

Components and distribution lines should be sloped and fitted with drain points so that the system can be completely drained. In distribution systems, dead legs and low-flow conditions should be avoided, and valved tie-in points should have length-to-diameter ratios of six or less. In systems that operate at self-sanitizing temperatures, precautions should be taken to avoid cool points where biofilm development could occur. If drainage of components or distribution lines is intended as a microbial control strategy, they should also be configured to be dried completely using dry compressed gas because drained but still moist surfaces will still support microbial proliferation. Water exiting from the distribution system should not be returned to the system without first passing through all or a portion of the purification system.

The distribution design should include the placement of sampling valves in the storage tank and at other locations, such as in the return line of the recirculating water system. Direct connections to processes or auxiliary equipment should be designed to prevent reverse flow into the controlled water system. Hoses and heat exchangers that are attached to points of use to deliver water must not chemically or microbiologically degrade the water quality. The distribution system should permit sanitization for microorganism control. The system may be continuously operated at sanitizing conditions or sanitized periodically.

5.1.14 NOVEL/EMERGING TECHNOLOGIES

New water treatment technologies are being developed continuously. Before these technologies are utilized in pharmaceutical water systems, they should be evaluated for acceptable use in a GMP environment. Other considerations should include the treatment process, reliability and robustness, use of added substances, materials of construction, and ability to validate. Consideration should be given to recognize the areas of concern during the evaluation and to identify control measures for the technology. This should include impact on chemical and microbial attributes.
5.2 Installation, Materials of Construction, and Component Selection

Installation techniques are important because they can affect the mechanical, corrosive, and sanitary integrity of the system. Valve installation should promote gravity drainage. Pipe supports should provide appropriate slopes for drainage and should be designed to support the piping adequately under worst-case thermal and flow conditions. The methods of connecting system components—including units of operation, tanks, and distribution piping—require careful attention to preclude potential operational and microbial problems.

Stainless steel welds should provide reliable joints that are internally smooth and corrosion-free. Low-carbon stainless steel, compatible wire filler where necessary, inert gas, automatic welding machines, and regular inspection and documentation help to ensure acceptable weld quality. Follow-up cleaning and passivation of metal surfaces after installation are important for removing contamination and corrosion products and to re-establish the passive corrosion-resistant surface.

Plastic materials can be fused (welded) in some cases, and also require smooth, uniform internal surfaces. Adhesive glues and solvents should be avoided due to the potential for voids and organic extractables. Mechanical methods of joining, such as flange fittings, require care to avoid the creation of offsets, gaps, penetrations, and voids. Use of plastic materials may contribute to TOC levels.

Control measures include good alignment, properly sized gaskets, appropriate spacing, uniform sealing force, and the avoidance of threaded fittings.

Materials of construction should be selected to be compatible with control measures such as sanitizing, cleaning, or passivation. Temperature rating is a critical factor in choosing appropriate materials because surfaces may be required to handle elevated operating and sanitization temperatures. If chemicals or additives will be used to clean, passivate, or sanitize the system, materials resistant to these chemicals or additives must be utilized. Materials should be capable of handling turbulent flow and elevated velocities without erosion of the corrosion-resistant film (such as the passive chromium oxide surface of stainless steel) or reduction in wall thickness for plastics. The finish on metallic materials such as stainless steel, whether it is a refined mill finish, polished to a specific grit, or an electropolished treatment, should complement the system design and provide satisfactory corrosion and microbial activity resistance. The finish should also be a material that can be chemically sanitized. Auxiliary equipment and fittings that require seals, gaskets, diaphragms, filter media, and membranes should exclude materials that permit the possibility of extractables, shedding, and microbial activity. Insulating materials exposed to stainless steel surfaces should be free of chlorides to avoid the phenomenon of stress corrosion cracking that can lead to system contamination and the destruction of tanks and critical system components.

Specifications are important to ensure proper selection of materials and to serve as a reference for system qualification and maintenance. Information such as the manufacturer’s metallurgical reports for stainless steel and reports of composition, ratings, and material-handling capabilities for nonmetallic substances should be reviewed for suitability and retained for reference.

Component (auxiliary equipment) selection should be made with assurance that it does not create a source of contamination and material-handling capabilities for nonmetallic substances should be reviewed for suitability and retained for reference.

Temperatures of 65°–80° are most commonly used for thermal sanitization. Continuously recirculating water of at least 65° at the coldest location in the distribution system has also been used effectively in stainless steel distribution systems when attention is paid to uniformity and distribution of such self-sanitizing temperatures. These techniques are limited to systems that are compatible with the higher temperatures needed to achieve sanitization. Frequent use of thermal sanitization at appropriate temperatures should eliminate the need for other sanitization methods.

The use of thermal methods at temperatures above 80° is contraindicated because it does not add to microbial control of the system or reduction of biofilm. Some methods (e.g., steam sanitizing, hot water circulation at temperatures ≥100°) can be less effective or even destructive because of the need to eliminate condensate or manipulate system components, stress materials of construction, deform filters, and its adverse impact on instrumentation.

Although thermal methods control biofilm development by either continuously inhibiting its growth or, in intermittent applications, by killing the microorganisms within developing biofilms, they are not effective in removing established biofilms. Killed but intact biofilms can become a nutrient source for rapid biofilm regrowth after the sanitizing conditions are removed or halted. In cases of infrequent thermal sanitizations that allow biofilm development between treatments, a combination of routine thermal treatment and periodic supplementation with chemical sanitization may be more effective. The more frequent the thermal sanitization, the more likely it is that biofilm re-development can be eliminated.

5.3 Sanitization

Microbial control in water systems is achieved primarily through sanitization practices. Systems can be sanitized using either thermal or (photo-)chemical means.

5.3.1 THERMAL SANITIZATION

Thermal approaches to system sanitization include periodic or continuously circulating hot water and the use of steam. Temperatures of 65°–80° are most commonly used for thermal sanitization. Continuously recirculating water of at least 65° at the coldest location in the distribution system has also been used effectively in stainless steel distribution systems when attention is paid to uniformity and distribution of such self-sanitizing temperatures. These techniques are limited to systems that are compatible with the higher temperatures needed to achieve sanitization. Frequent use of thermal sanitization at appropriate temperatures should eliminate the need for other sanitization methods.

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5.3.2 CHEMICAL SANITIZATION

Chemical methods, where compatible, can be used on a wider variety of construction materials. These methods typically use oxidizing agents such as ozone, hydrogen peroxide, peracetic acid, or combinations thereof. Halogenated compounds can be effective sanitizers but are less aggressive oxidizing agents and may be difficult to flush from the system. Chemical agents may not penetrate the full biofilm matrix or extend into all biofilm locations (such as crevices at gasketed fittings) and may...
leave biofilms incompletely inactivated. Compounds such as ozone, hydrogen peroxide, and peracetic acid oxidize bacteria and biofilms with reactive peroxides and by forming very reactive free radicals (notably hydroxyl radicals). The short half-life of ozone in particular, and its limitation on achievable concentrations, require that it be added continuously during the sanitization process. Hydrogen peroxide and ozone rapidly degrade to water and/or oxygen, and peracetic acid degrades to oxygen and acetic acid. The ease of degradation of ozone to oxygen using 254-nm UV lights in circulating loops allows it to be used effectively on a continuously sanitizing basis in holding tanks and on an intermittent basis (e.g., daily or weekly) in the distribution loops. The highly reactive nature of ozone requires the use of system materials and components that are even more oxidation resistant than those typically used with the other oxidizing agents.

It is important to note that microorganisms in a well-developed biofilm can be extremely difficult to kill, even by using aggressive oxidizing chemicals. The less developed and therefore thinner the biofilm, the more effective the biofilm inactivation. Therefore, optimal microbial control is achieved by using oxidizing chemicals at a frequency that does not permit significant biofilm development between treatments.

Validation of chemical sanitization requires demonstration of adequate chemical concentrations throughout the system, exposure to all wetted surfaces including the body of use point valves, and complete removal of the sanitant from the system at the completion of treatment. Methods validation for the detection and quantification of residues of the sanitant or its objectionable degradants is an essential part of the validation program.

5.3.3 UV SANITIZATION

In-line UV light at a wavelength of 254 nm can also be used to continuously “sanitize” only the water circulating in the system, but these devices must be properly sized for the water flow. Such devices inactivate a high percentage (but not 100%) of microorganisms that flow through the device but cannot be used to directly control existing biofilm upstream or downstream of the device. However, when coupled with conventional thermal or chemical sanitization technologies or located immediately upstream of a microbiologically retentive filter, UV light is most effective and can prolong the interval between needed system re-sanitizations.

5.3.4 SANITIZATION PROCEDURES

Sanitization steps require validation to demonstrate the ability to reduce and hold microbial contamination at acceptable levels. Validation of thermal methods should include a heat distribution study to demonstrate that sanitization temperatures are achieved throughout the system, including the body of use point valves; sampling ports; instrument side branches; and fittings, couplings, and adapters, relying on water convection and thermal conduction through system materials for heat transfer to wetted surfaces.

The routine frequency of sanitization should be supported by the results of system microbial monitoring. Conclusions derived from trend analysis of the microbiological data should be used as the alert mechanism for the need for extraordinary maintenance. The routine frequency of sanitization should be established in such a way that the system operates in a state of microbiological control and does not regularly exceed Alert and Action Levels (see 9.4 Defining Alert and Action Levels and Specifications).

5.4 Operation, Maintenance, and Control

A preventive maintenance program should be established to ensure that the water system remains in a state of control. The program should include 1) procedures for operating the system, 2) monitoring programs for critical quality attributes and operating conditions including calibration of critical instruments, 3) schedule for periodic sanitization, 4) preventive maintenance of components, and 5) control of changes to the mechanical system and to operating conditions.

5.4.1 OPERATING PROCEDURES

Operating procedures for the water system and for performing routine maintenance and corrective action should be written, and they should also define the point when action is required. The procedures should be well documented, and should detail the function of each job, assign who is responsible for performing the work, describe how the job is to be done, and identify acceptable operating parameters. The effectiveness of these procedures should be assessed during water system validation.

5.4.2 PROCESS MONITORING PROGRAM

A process-monitoring program should establish the critical quality attributes and operating parameters that are documented and monitored. The program may include a combination of in-line sensors and/or automated instruments (e.g., for temperature, TOC conductivity, hardness, and chlorine), automated or manual documentation of operational parameters (e.g., flow rates or pressure drop across a carbon bed, filter, or RO unit), and laboratory tests (e.g., total microbial counts). The frequency of sampling, the requirement for evaluating test results, and the necessity of initiating corrective action should be included.

5.4.3 ROUTINE MICROBIAL CONTROL

Sanitization may be integral to operation and maintenance, and necessary on a routine basis, depending on system design and the selected units of operation, to maintain the system in a state of microbial control. Technologies for sanitization are described above in more detail in 3.3 Sanitization.
5.4.4 PREVENTIVE MAINTENANCE

A preventive maintenance program should be in effect. The program should establish what preventive maintenance is to be performed, the frequency of maintenance work, and how the work should be documented.

5.4.5 CHANGE CONTROL

The mechanical configuration, operating conditions, and maintenance activities of the water system must be controlled. Proposed changes should be evaluated for their impact on the whole system. The need to realign the system after changes are made should be determined. After a decision is made to modify a water system, the affected drawings, manuals, and procedures should be revised. Portions or operations of the water system that are affected by the modification should be tested to demonstrate a continued state of control. The extent and duration of testing should be related to the risk impact of the change to the system.

Change to read:

6. SAMPLING

The testing of water samples from a water system is critical to the ongoing control of the system and assessment of the quality of the water being used. If improperly collected, a sample could yield a test result that is unrepresentative of the sample’s purpose. This could lead to inaction when remediation is needed or to unnecessary remediation when none is necessary. It could also lead to misinterpretations of product impact. Therefore, properly collecting water samples, understanding their purpose, and establishing appropriate water system sampling plans are essential to water quality control and system control.

6.1 Purposes and Procedures

To assess a particular water attribute testing, a sample of the water usually must be removed from a water system for specific attribute testing. The sample needs to be obtained from specific locations that are representative for the purpose being monitored. This sample may be analyzed by in-line/on-line instruments or it may be completely removed from the system as a “grab sample” in a container for off-line testing. In-line/on-line testing avoids the exogenous contamination potential of grab samples that could lead to artifactually variable data trends and incorrect decisions on system performance, maintenance, and utilized as well as initiating fruitless causative investigations. Grab samples may be appropriate where the water in the system is not homogeneous for certain attributes.

The data from water testing are generally used for one of two purposes: for process control (PC) of the water purification and distribution system or for QC of the water being drawn from the system for some application or use. In many cases, depending on the sampling location and sampling process, the resulting data can be used for both PC and QC purposes.

6.1.1 PC SAMPLING

Because PC sampling is intended to reflect the quality of the water behind the valve and within the distribution system, coming from the purification system, or between its purification steps, efforts should be made to avoid contaminating the water as it is drawn from the system so that its test results accurately reflect the water quality within the system at that location. This may require the use of strategically located sampling ports, in addition to points of use.

If microbial testing is needed for PC purposes, the sampling valve should have a properly installed, sanitary design that uses vigorous pre-sampling flushing. This flushing shears off fragile biofilm structures growing on surfaces within the valve and water path before the sample is collected. This avoids biasing the microbial count of perhaps pristine water in the system behind that valve. A fully open valve flush (at >8 ft/s velocity within the valve and connector) for at least 30 s typically provides sufficient shear forces to adequately remove any fragile biofilm structures. Additional control measures for preventing sample contamination could also include stringent pre- and post-sampling outlet sanitation, the use of sterile hoses and gaskets or other connectors to direct the water flow, and other measures.

The data from PC sampling indicate how well the system is maintaining the water quality at that sampling location. These data are subsequently used to signal when some extraordinary intervention might be needed, in addition to normal maintenance and system sanitization operations, to restore the system to the expected level of purity.

PC sampling can only be used to indicate the quality of the water being delivered to the points of use (for QC purposes) if it has been shown to be representative of that point-of-use quality. This may be possible with chemical attributes that are typically not affected by the fluid path of the water delivery process, but is generally not possible with microbial attributes, which can be greatly affected by localized biofilms along that fluid path. If this fluid path is not utilized for PC sampling, then the resulting data typically cannot be used for QC purposes.

6.1.2 QC SAMPLING

QC sampling is intended to reflect the quality of water that is being used. These samples should be collected at the true point of use; that is, where the water is delivered for use, not where it leaves the water system. QC sampling must utilize that same delivery path and components utilized for a water transfer during actual water use. This includes the same valves, hoses, heat exchangers, flow totalizers, hard-piped connections, and other components utilized during water use.

In addition to the water transfer components, QC sampling must also use the same water transfer process employed during water use, including the same pre-use outlet and delivery path flushing procedure and the same outlet, fitting, and hose
sanitization practices employed during actual water use. The water delivery process and components used for QC sampling must be identical to manufacturing practices at every system outlet for the QC sample to mimic the quality of water being used by accumulating the same chemical and microbial contaminant levels it would during actual use from that outlet location. Where permanent connections from the water system to equipment are present, accommodation should be made in the design to collect samples from locations as close to the equipment as possible. For example, samples can be collected from special sample ports or other valves near the equipment connection that allow the collected water sample to accurately reflect the water quality that is used. Where the water transfer conduit is designed and/or definitively treated to eliminate all contaminating influences prior to water transfer through that conduit, PC sampling locations within the distribution system can reflect the quality of the water that is actually used for QC purposes at those permanent connections. However, the success of the design and treatments intended to eliminate these contaminating influences must be verified. This is typically done during water system validation.

Where routine water use practices involve contamination-prone activities, such as no pre-use flushing or poor hose/stationary practices, these water use practices should be improved to reduce the potential for delivering contaminated water from the water system and for unacceptable QC sample testing results that reflect that same contamination.

### 6.2 Attributes and Sampling Locations

The tests being performed on the samples are relevant to the sampling location and purpose of the sample. In-process monitoring of nonmonograph attributes may be indicated for specific unit operations. For instance, before and after a softener, it may be important to determine water hardness to verify softener efficacy. Before and after an activated carbon bed/filter, it may be important to verify chlorine or TOC removal and/or reduction or test for an increase in microbial count. Before a distillation unit, it may be important to quantify the incoming bacterial endotoxin level to ensure that the still is not being over-challenged beyond its typical 3–4 log purification capability. However, once the water is in the distribution system, the compendial attributes of importance typically include at least conductivity, TOC, and microbial count. In Water for Injection systems and other systems or system locations where bacterial endotoxin control is important, endotoxin is also assayed. Other tests may be necessary depending on the intended uses of the water.

#### 6.2.1 CHEMICAL ATTRIBUTES

Dissolved chemical contaminants detected by conductivity or TOC testing tend to be uniformly distributed in the water throughout the water system. However, there are exceptions where localized chemical contamination sources can occur, such as from a coolant-leaking heat exchanger in a sub-loop, or at a point of use, or within a dead leg. These chemical contaminants may only be seen at the associated outlets and not systemically. However, in the absence of localized contamination influences, chemical attributes are candidates for on-line testing at fixed strategic locations within the distribution system, such as near a circulating loop return, and are generally reflective of the same chemical quality at all locations and points of use within the distribution system. Nevertheless, the suitability of the on-line locations of these instruments for QC release purposes must be verified as being representative of the use-point water quality. This is usually done during water system validation.

#### 6.2.2 MICROBIAL ATTRIBUTES

The same uniformity scenario cannot be assumed for microbial attributes. Planktonic organisms in a water sample could have originated from biofilms in the purification or distribution systems releasing more or less uniform levels of planktonic organisms into the circulating water, as detectable in samples from all outlets. However, a local biofilm developing within a water delivery conduit (such as a use-point outlet valve and transfer hose) in an otherwise pristine biofilm-free water system could release planktonic organisms detectable only in water delivered through that conduit. Therefore, QC release samples for assessing the quality of water that is delivered by the system during water use must be collected after the water has traversed the same fluid conduit (including the same preparatory activities such as outlet sanitization and pre-flushing) from the water distribution system to the specific locations where the water is used.

On-line microbial water sampling/testing has value in pharmaceutical water systems only for PC purposes unless the water is taken from the point of use in the same manner as routine water usage, in which case the data can also have a QC release purpose. Microbial counts detected from strategic sampling ports continue to have PC and investigational value, but generally cannot be substituted for QC release testing except in certain scenarios, as described in 6.1.2 QC Sampling.

### 6.3 Validation Sampling Plans

The initial sampling plan for a pharmaceutical water system is usually developed for a validation program (see 4. Validation

and Qualification of Water Purification, Storage, and Distribution Systems). This strategy is for characterization of the system’s ability to purify, distribute, and deliver pharmaceutical water. Typically, the initial validation sampling is for a short duration (e.g., at least 2–4 weeks) at a high sampling frequency to generate a significant body of data that will allow detection of short-term or localized chemical or microbial quality deviations from all outlets. These data provide an initial assessment of system performance to guide decisions about using the water for operational purposes.

The initial validation sampling plan is re-evaluated when the pharmaceutical water is placed into operation, typically to reduce the amount of data being generated while not compromising the ability to identify anomalous operations/events, especially during the early life cycle of the water system. In the absence of such quality deviations during the initial sampling period, the sampling frequency can be lessened for a period of time (e.g., at least 2–4 additional weeks) to ensure that somewhat longer-term adverse quality trends are not apparent. During this second period of time, the water may be considered for at-risk routine use, pending the acceptable completion of the second validation sampling period. After successful completion, monitoring can eventually be lessened again to what will become the routine sampling plan.
Periodic review of the water system operation and monitoring needs to be performed to assess seasonal source water variability, effectiveness of sanitization, and routine maintenance events. Periodic review should be performed during the complete life cycle of the water system, typically annually, for evidence of longer-term data trends and quality deviations. The routine sampling plan should be re-evaluated periodically based on the available data to determine the appropriate frequency and sample locations. This review offers an opportunity to improve data evaluation and reduce workloads based on what that data indicate relative to process and quality control. The routine sampling plan should have a rationale for the frequency and locations that are selected to justify how the resulting data will be used to characterize the overall operation of the system and the release of the water for use.

6.4 Routine Sampling Plans

6.4.1 SOURCE WATER SAMPLING

As mentioned in earlier sections, the source water for pharmaceutical water systems must comply with the standards for one of the Drinking Waters listed in the associated compendial water monograph or in General Notices. When a municipality or other water authority is providing this Drinking Water, they are required to comply with the local Drinking Water Regulations for the water supplied to a drinking or potable water distribution piping grid for that region. The quality of that water by the time it reaches the pharmaceutical user is dependent on a number of factors including distance from the input source, duration of travel within the piping, and condition of the piping in that potable water distribution grid, any of which could have adversely affected some of its initial chemical and/or microbial attributes. Based on a risk assessment, it may be prudent to verify full compliance with regulations using water collected from sample ports prior to the pretreatment system, or other equivalent Drinking Water outlets within the facility. If the water complies, then continued assurance of compliance could be verified using Drinking Water Regulation test results provided by the water authority or by periodic retesting of selected or all the Drinking Water attributes by the user or by both the user and the water authority. If private sourced water is utilized, it is the user’s responsibility to demonstrate full Drinking Water regulation compliance, using water samples from such sampling ports on a periodic basis as determined by a risk analysis.

These pre-treatment sampling ports could, at the user’s discretion, be used to periodically monitor other source water attributes that could affect specific pretreatment or purification unit operations. Depending on the user’s source water quality consistency and a risk assessment of its potential impact on the purification process, the periodically monitored attributes could include microbial count, absence of coliforms, bacterial endotoxin levels, conductivity, TOC, pH, hardness, chlorine, silica, turbidity or silt density index, and others. These data could be useful in investigations and for operational adjustments to critical unit operation parameters and maintenance procedures, or for feedback to the potable water provider if unusual trends are observed.

6.4.2 PRETREATMENT AND PURIFICATION SYSTEM SAMPLING

The location and frequency of sampling from ports within the pretreatment and purification systems may be selected based on a risk analysis of unit operation purpose. The purpose of this sampling is primarily for PC, for example, to ensure maintenance of acceptable unit operation performance, to assess maintenance procedure efficacy, and to investigate the need for remedial action. Quality deviations in the early portions of the purification process can affect unit operation efficiency but usually do not impact the finished water quality or acceptable use.

6.4.3 PURIFIED WATER DISTRIBUTION SYSTEM SAMPLING

Purified Water distribution system sampling is intended to provide continuing assurance of ongoing PC and compliance with the user’s finished water chemical and microbiological requirements. Generally, the locations for that sampling and the frequency of testing the specific attributes are a matter of process and quality control consistency, as well as risk tolerance in the event of a deviation.

Depending on the water system design, the chemical attributes of a water system tend to be relatively constant and more uniformly distributed than the microbiological attributes. Therefore, less frequent sampling at only selected locations could be justified for chemical testing based on familiarity with system design and the existence of historically consistent operational data. However, with some purification system designs, the chemical quality could change dramatically in a short period of time (such as from the exhaustion of deionization beds), so frequent or even continuous in-line/on-line monitoring of the chemical attributes would be advisable to be able to recognize and correct the cause of the problem before non-compliant water is produced and used.

For microbial testing, all use points and critical sample ports in a distribution system are typically sampled routinely, including those that are infrequently used by manufacturing. There is no prescribed sampling frequency for Purified Water system outlets, so typical outlet sampling frequencies vary from daily to monthly, with sampling occurring somewhere in the system at least at weekly intervals.

A risk analysis is suggested for determining the sampling plan for a Purified Water system. Factors in this analysis could include (but are not limited to) the test result history for the entire water system as well as specific outlets, the criticality of specific outlets to manufacturing, the usefulness of selected sample ports as indicators of ongoing system control, and the scope of impact on products and activities should an unfavorable test result occur. For the scope of impact, the less frequent the sampling, the more products and processes will be impacted by an unfavorable test result.

6.4.4 WATER FOR INJECTION DISTRIBUTION SYSTEM SAMPLING

The sampling plans for Water for Injection distribution systems (as well as any water system where some level of bacterial endotoxin control is needed) utilize the same general sampling approaches as do Purified Water systems. However, the
regulatory expectations for Water for Injection distribution system sampling plans are more prescriptive because microbial control must be much more stringent as it is related to the bacterial endotoxin attribute. In general, water sampling for microbial and bacterial endotoxin testing is expected to occur daily somewhere in the system, with each outlet being sampled periodically, based on a risk assessment, to characterize the quality of the water.

6.5 Non-Routine Sampling

Non-routine sampling can also be performed on the water system for episodic events or reasons for which the routine sampling plans are insufficient to capture the needed information. Examples include change control purposes such as evaluating potential changes to sampling, testing, maintenance procedures, or system design; data or event excursion investigation purposes; or simply for long-term informational purposes and establishing baselines for future investigational value. The purpose of the non-routine sampling dictates the sampling procedures to be used, the attributes to be tested, and the location and repeating occurrence (if any) of that testing. It should also be noted that such non-routine sampling may be done from sampling ports that may or may not be routinely tested. Sampling ports can be positioned in a water system purely for investigational, non-routine sampling, and as such, they do not need to be part of a routine sampling plan.

**Change to read:**

7. CHEMICAL EVALUATIONS

7.1 Chemical Tests for Bulk Waters

The chemical attributes of Purified Water and Water for Injection that were in effect prior to USP 23 were specified by a series of chemistry tests for various specific and nonspecific attributes with the intent of detecting chemical species indicative of incomplete or inadequate purification. Although these methods could have been considered barely adequate to control the quality of these waters, they nevertheless stood the test of time. This was partly because the operation of water systems was, and still is, based on on-line conductivity measurements and specifications generally thought to preclude the failure of these archaic chemistry attribute tests.

In 1996, USP moved away from these chemical attribute tests, switching to contemporary analytical technologies for the bulk waters Purified Water and Water for Injection. The intent was to upgrade the analytical technologies without tightening the quality requirements. The two contemporary analytical technologies employed were TOC and conductivity. The TOC test replaced the test for Oxidizable Substances that primarily targeted organic contaminants. A multi-staged conductivity test that detects ionic (mostly inorganic) contaminants replaced, with the exception of the test for Heavy Metals, all of the inorganic chemical tests (i.e., Ammonia, Calcium, Carbon Dioxide, Chloride, Sulfate).

Replacing the heavy metals attribute was considered unnecessary because 1) the source water specifications (found in the U.S. EPA’s NPDWR) for individual heavy metals were tighter than the approximate limit of detection of the Heavy Metals test for USP XXII Water for Injection and Purified Water (approximately 0.1 ppm), 2) contemporary water system construction materials do not leach heavy metal contaminants, and 3) test results for this attribute have uniformly been negative; there has not been a confirmed occurrence of a singular test failure (failure of only the Heavy Metals test with all other attributes passing) since the current heavy metal drinking water standards have been in place. Total Solids and pH were the only tests not covered by conductivity testing. The test for Total Solids was considered redundant because the nonselective tests of conductivity and TOC could detect most chemical species other than silica, which could remain undetected in its colloidal form. Colloidal silica in Purified Water and Water for Injection is easily removed by most water pretreatment steps, and even if present in the water, it constitutes no medical or functional hazard except in extreme and rare situations. In such extreme situations, other attribute extremes are also likely to be detected. It is, however, the user’s responsibility to ensure fitness for use. If silica is a significant component in the source water, and the purification unit operations could fail and selectively allow silica to be released into the finished water (in the absence of co-contaminants detectable by conductivity), then either silica-specific testing or a total-solids type testing should be utilized to monitor for and control this rare problem.

The pH attribute was eventually recognized to be redundant to the conductivity test (which included pH as an aspect of the test and specification); therefore, pH was discontinued as a separate attribute test. The rationale used by USP to establish its Purified Water and Water for Injection conductivity specifications took into consideration the conductivity contributed by the two least-conductive former attributes of Chloride and Ammonia, thereby precluding their failure had those wet chemistry tests been performed. In essence, the Stage 3 conductivity specifications (see Water Conductivity (645), Bulk Water, Procedure, Stage 3) were established from the sum of the conductivities of the limit concentrations of chloride ions (from pH 5.0 to 6.2) and ammonia ions (from pH 6.3 to 7.0), plus the unavoidable contribution of other conductivity-contributing ions from water (H+ and OH–), dissolved atmospheric carbon dioxide (as HCO3–), and an electro-balancing quantity of either sodium (Na+) or chlorine (Cl–), depending on the pH-induced ionic imbalance (see Table 1). The Stage 2 conductivity specification is the lowest value in this table, 2.1 μS/cm. The Stage 1 specifications, designed primarily for on-line measurements, were derived by essentially summing the lowest values in a group (H+, OH–, HCO3–, and Na+) of contributing ion columns for each of a series of tables similar to Table 1, created for each 5° increment between 0° and 100°. For example purposes, the italicized values in Table 1, the conductivity data table for 25°, were summed to yield a conservative value of 1.3 μS/cm, the Stage 1 specification for a nontemperature-compensated, nonatmosphere-equilibrated water sample that actually had a measured temperature of 25°–29°. Each 5° increment in the table was similarly treated to yield the individual values listed in the table of Stage 1 specifications (see Water Conductivity (645), Bulk Water).
Table 1. Contributing Ion Conductivities of the Chloride-Ammonia Model as a Function of pH (in atmosphere-equilibrated water at 25°C)

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<th>H⁺</th>
<th>OH⁻</th>
<th>HCO₃⁻</th>
<th>Cl⁻</th>
<th>Na⁺</th>
<th>NH₄⁺</th>
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<td>1.22</td>
<td>4.63</td>
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As stated above, this rather radical change to utilizing a conductivity attribute as well as the inclusion of a TOC attribute allowed for on-line measurements. This was a major philosophical change and allowed industry to realize substantial savings. The TOC and conductivity tests can also be performed off-line in the laboratories using collected samples, although sample collection tends to introduce opportunities for adventitious contamination that can cause false high readings. The collection of on-line data is not, however, without challenges. The continuous readings tend to create voluminous amounts of data, where previously only a single data point was available. As stated in 6. Sampling, continuous in-process data are excellent for understanding how a water system performs during all of its various usage and maintenance events in real time, but this is too much data for QC purposes. Therefore, for example, one can use a justifiable portion of the data (at a designated daily time or at the time of batch manufacturing) or the highest value in a given period as a worst case representation of the overall water quality for that period. Data averaging is generally discouraged because of its ability to obscure short-lived extreme quality events.

7.2 Chemical Tests for Sterile Waters

Packaged/sterile waters present a particular dilemma relative to the attributes of conductivity and TOC. The package itself is the major source of chemicals (inorganics and organics) that leach over time into the packaged water and can easily be detected by the conductivity and TOC tests. The irony of organic leaching from plastic packaging is that before the advent of bulk water TOC testing, when the Oxidizable Substances test was the only “organic purity” test for both bulk and packaged/sterile water monographs in USP, the insensitivity of that test to many of the organic leachables from plastic and elastomeric packaging materials was largely unrecognized, allowing organic levels in packaged/sterile water to be quite high (possibly many times the TOC specification for bulk water).

Similarly, glass containers can also leach inorganics, such as sodium, which are easily detected by conductivity but poorly detected by the former wet chemistry attribute tests. Most of these leachables are considered harmless based on current perceptions and standards at the rather significant concentrations present. Nevertheless, they effectively degrade the quality of the high-purity waters placed into these packaging systems. Some packaging materials contain more leachables than others and may not be as suitable for holding water and maintaining its purity.
The attributes of conductivity and TOC tend to reveal more about the packaging leachables than they do about the water's original purity. These currently "allowed" leachables could render the sterile packaged versions of originally equivalent bulk water essentially unsuitable for many uses where the bulk waters are perfectly adequate. Therefore, to better control the ionic packaging leachables, (645) is divided into two sections. The first, Water Conductivity (645), Bulk Water, applies to Purified Water, Water for Injection, Water for Hemodialysis, and Pure Steam, and includes the three-stage conductivity testing instructions and specifications. The second, Water Conductivity (645), Sterile Water, applies to Sterile Purified Water, Sterile Water for Injection, Sterile Water for Inhalation, and Sterile Water for Irrigation. The Sterile Water section includes conductivity specifications similar to the Water Conductivity (645), Bulk Water, Procedure, Stage 2 testing approach because it is intended as a laboratory test, and these sterile waters were made from bulk water that already complied with the three-stage conductivity test. In essence, packaging leachables are the primary target analytes of the conductivity specifications in Water Conductivity (645), Sterile Water. The effect on potential leachables from different container sizes is the rationale for having two different specifications, one for small packages containing nominal volumes of 10 mL or less and another for larger packages. These conductivity specifications are harmonized with the European Pharmacopoeia conductivity specifications for Sterile Water for Injection. All monographed waters, except Bacteriostatic Water for Injection, have a conductivity specification that directs the user to either the Bulk Water or the Sterile Water section. For the sterile packaged water monographs, this water conductivity specification replaces the redundant wet chemistry limit tests intended for inorganic contaminants that had previously been specified in these monographs.

Controlling the organic purity of these sterile packaged waters, particularly those in plastic packaging, is more challenging. Although the TOC test can better detect these impurities and therefore can be better used to monitor and control these impurities than the current Oxidizable Substances test, the latter has a history of use for many decades and has the flexibility to test a variety of packaging types and volumes that are applicable to these sterile packaged waters. Nevertheless, TOC testing of these currently allowed sterile, plastic-packaged waters reveals substantial levels of plastic-derived organic leachables that render the water perhaps orders of magnitude less organically pure than is typically achieved with bulk waters. Therefore, usage of these packaged waters for analytical, manufacturing, and cleaning applications should only be exercised after the purity of the water for the application has been confirmed as suitable.

7.3 Storage and Hold Times for Chemical Tests

Due to the homogeneous nature of chemical impurities in water, unlike the challenges of microbial impurities, the storage requirements and impact of holding times are very practically determined. In general, the chemical purity of high-purity water samples can only degrade over time, possibly generating a failed result of the sample that would have passed if it were tested immediately or on-line. The general fact is that the longer samples are stored, the greater the potential to be adversely impacted by containers or conditions.

For off-line chemical tests of waters, there are no compendial requirements for storage time and conditions. However, the general recommendation is to perform testing as soon as practical to avoid false adverse results. Where possible, store cool and measure as quickly as practical. This reduces the chances that a water sample gets contaminated over time, and this would reduce unwarranted and unnecessary investigations of false positives.

7.3.1 Containers

When sampling water for off-line analysis, the selection and cleanliness of the container play a significant part in obtaining accurate data. For samples to be tested for chemical impurities according to (645) and Total Organic Carbon (643), the proper container should be one that does not contaminate the sample during the storage/hold time. For example, the use and preparation of glass containers could be very acceptable for storing samples for TOC testing, but some glass containers do leach ions over time (hours and days), and they can adversely impact a conductivity test by creating a false positive result—if the storage time is too long. Likewise, there are some polymer materials that can adversely impact the TOC chemical impurity in water. However, many polymer materials are very inert. In any case, cleanliness of the container is crucial because trace quantities of soaps and fingerprints will adversely impact the chemical purity of the water. Properly cleaned containers are acceptable because chemical impurities are easily rinsed away. Extensive chemical cleaning methods such as acid or caustic rinsing should never be needed. If they are needed, consider replacing the containers.

7.3.2 Storage Time and Conditions

There are no specific recommendations for storage of samples for water analyses. If there is some trace interaction of the container and water, then generally colder and shorter storage times are better than warmer and longer storage times. Chemical dissolution and reactivity are usually enhanced by increased temperature. Furthermore, time is always an element because the water sample can only get worse in a container, and it never gets better with time.

7.4 Elemental Impurities in Pharmaceutical Waters

Elemental impurities (EI) have the most restrictive limits for Water for Injection used in manufacturing parenterals, in particular large-volume injections (see Injections and Implanted Drug Products (1) for a definition of large-volume injections) because of the large dose. The most restrictive permissible daily exposure (PDE) of EI resides with lead, mercury, cadmium, and arsenic. Other EI listed in (232) permit a substantially higher PDE, and are therefore less restrictive.

Water that meets U.S. EPA National Primary Drinking Water Regulations or WHO Drinking Water Guidelines that has been purified by conventional technologies used to produce Water for Injection can comply with (232) for parenterals.

Published on March 26, 2020
Table 2 shows that source water that meets US EPA NPDR or WHO Drinking Water Guidelines has maximum contaminant levels (concentration) for lead, mercury, cadmium, and arsenic that are NMT 10 times (1-log) higher than the EI limits for parenterals, based on a daily dose of 2000 mL. For a smaller volume injection, the allowed parenteral daily dose of EI is correspondingly higher. The purification technologies needed to produce Water for Injection that reduce the impurities by a factor of 100 to 1000 will assure compliance with (232), provided there are no elemental impurities added during processing, packaging, delivery, or storage.

<table>
<thead>
<tr>
<th>Element</th>
<th>Parenteral PDE (µg/day)</th>
<th>Parenteral Daily Dose (µg/mL a)</th>
<th>U.S. EPA National Primary Drinking Water Regulations (µg/mL b)</th>
<th>WHO Drinking Water Guidelines (µg/mL c)</th>
<th>Result of 2-Log Reduction of EI Concentration for WFI (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cadmium</td>
<td>2</td>
<td>0.001</td>
<td>0.005</td>
<td>0.003</td>
<td>0.00005</td>
</tr>
<tr>
<td>Lead</td>
<td>5</td>
<td>0.0025</td>
<td>0.015</td>
<td>0.01</td>
<td>0.00015</td>
</tr>
<tr>
<td>Inorganic arsenic</td>
<td>15</td>
<td>0.0075</td>
<td>0.01</td>
<td>0.01</td>
<td>0.0001</td>
</tr>
<tr>
<td>Inorganic mercury</td>
<td>3</td>
<td>0.0015</td>
<td>0.002</td>
<td>0.006</td>
<td>0.00006</td>
</tr>
</tbody>
</table>

a Concentration based on a daily dose of 2000 mL, and all drug product elemental impurities coming from the water component.
b Drinking Water Regulations state these Maximum Contaminant Levels (MCLs) as mg/L, which equals µg/mL or ppm.
c Determined from the greater of the US EPA Regulations column and WHO Guidelines column for each element, then divided by 100 (2-log).

Chemical purification technologies for Purified Water are similarly efficient in removing EI as those for Water for Injection production. Because all sterile waters are prepared from Purified Water or Water for Injection, the assurance of compliance to (232) extends to sterile waters, provided there are no elemental impurities added during processing, packaging, delivery, or storage.

Further discussion can be found in Pharmacopeial Forum [see Bevilacqua A, Soli TC, USP Chemical Analysis Expert Committee. Elemental impurities in pharmaceutical waters. Pharm Forum. 2013;39(1)].

Change to read:

8. MICROBIAL EVALUATIONS

This section of the chapter presents a discussion about the types and sources of microorganisms and whether certain microbes are prone to colonize pharmaceutical water systems. This section also addresses microbiological examination of water samples, including a discussion on recovery methods.

8.1 Microorganism Types

Microorganisms are ubiquitous and their natural habitats are extremely diverse. Based on comparative ribosomal RNA sequencing, the phylogenetic tree of life consists of three domains: Bacteria and Archaea (both prokaryotes), and Eukarya (eukaryotes). Most microorganisms that contaminate pharmaceutical products are prokaryotic bacteria and eukaryotic fungi (yeasts and molds). These microbes are typical isolates from pharmaceutical environments, including the associated personnel, and a few are frank or opportunistic pathogens. Contamination with viruses is a concern in bioprocessing that uses animal cells.

8.1.1 ARCHEAENS

Microbes from the domain Archaea are phylogenetically related to prokaryotes but are distinct from bacteria. Many are extremophiles, with some species capable of growing at very high temperatures (hyperthermophiles) or in other extreme environments beyond the tolerance of any other life form. In general, most extremophiles are anaerobic or microaerophilic chemolithoautotrophs. Because of their unique habitats, metabolism, and nutritional requirements, Archaeans are not known to be frank or opportunistic pathogens, and they are not capable of colonizing a pharmaceutical water system.

8.1.2 BACTERIA

Bacteria are of immense importance because of their rapid growth, mutation rates, and ability to exist under diverse and adverse conditions; some of them are human pathogens. Some are very small and can pass through 0.2-µm rated filters. Others form spores, which are not part of their reproductive cycle. Bacterial spore formation is a complex developmental process that allows the organisms to produce a dormant and highly resistant cell in times of extreme stress. Bacterial endospores can survive high temperatures, strong UV irradiation, desiccation, chemical damage, and enzymatic destruction, which would normally kill vegetative bacteria.

Using a traditional cellular staining technique based on cell wall compositional differences, bacteria are categorized into Gram positive and Gram negative, although many sub-groups exist within each category based on genomic similarities and differences.

8.1.2.1 Gram-positive bacteria: Gram-positive bacteria are common in a pharmaceutical manufacturing environment but not in water systems. This is because they are generally not suited to surviving in a liquid environment that has the chemical purity of a pharmaceutical-grade water system. Gram-positive bacteria include the spore-forming bacteria from the genus Bacillus,
which are common soil and dust microorganisms and the non-sporulating bacteria from the genera *Staphylococcus*, *Streptococcus*, and *Micrococcus*, which normally colonize human skin and mucous membranes. Other types of Gram-positive bacterial microorganisms include organisms from the genera *Corynebacterium*, *Mycobacterium*, *Arthrobacter*, *Propionibacterium*, *Streptomyces*, and *Actinomyces*. This latter group of microbes can be found in various natural habitats including the human skin and soil.

Although Gram-positive bacteria can be detected in pharmaceutical water samples, their recovery is often associated with faulty aseptic technique during sampling or testing, or associated with exogenous contamination sources. These non-aquatic microorganisms could be present in source water and could, in rare circumstances, make their way into the early stages of a water purification unit operation. Gram-positive bacteria are not known to colonize water systems. In addition, these microbes will likely be removed by one or more of the purification unit operations prior to the ultimate creation of the pharmaceutical-grade water.

### 8.1.2 Gram-negative bacteria:

These types of bacteria are found in soil, water, plants, and animals. Gram-negative bacteria are relevant to pharmaceutical manufacturers, primarily due to their production of endotoxins as well as their ability to populate water systems, a topic discussed in 8.4 Endotoxin. Some Gram-negative bacteria prefer aquatic habitats and tend to colonize water systems and other wet environments as biofilms, a topic discussed in 8.2 Biofilm Formation in Water Systems.

### 8.1.2.3 Mycoplasma:

Organisms from the genus *Mycoplasma* are the smallest of the bacteria. Unlike other bacteria, these organisms do not have a cell wall and many exist as intracellular or animal/plant parasites. Mycoplasmas also require specific nutrients for survival, including sterols, and they cannot survive in a hypotonic environment such as pure water. Based on these facts, this type of bacteria is not a concern for pharmaceutical-grade water systems.

### 8.1.3 FUNGI

Fungi are mainly aerobic mesophilic microbes. They exist as unicellular (yeast) and multicellular filamentous (mold) organisms. Molds are often found in wet/moist but usually non-aquatic environments, such as soil and decaying vegetation. Yeasts are often associated with humans and vegetation, and both yeasts and molds also can be found in pharmaceutical environments. As mold matures it develops spores, which, unlike bacterial spores, are part of its reproductive cycle and are less resistant to adverse conditions. Mold spores are easily spread through air and materials, and could contaminate water samples.

Neither yeasts nor molds are suited for colonization or survival in pharmaceutical water systems. Their recovery is often associated with faulty aseptic technique during sampling or testing, or associated with exogenous contamination sources. These non-aquatic microorganisms, if present in source water, could make their way into the early stages of a water purification system; however, they will likely be removed by one or more of the purification unit operations.

### 8.1.4 VIRUSES

A virus is a small infectious agent unlike eukaryotes and prokaryotes. This is because viruses have no metabolic abilities of their own. Viruses are genetic elements containing either DNA or RNA that replicate within host cells. Human pathogenic viruses, especially those of fecal origin, could be present in source water. However, they are easily neutralized by typical water purification treatments, such as chlorination. Therefore, it is unlikely that human pathogenic viruses will be present or will proliferate (due to the absence of host cells) in pharmaceutical-grade waters.

### 8.1.5 THERMOPHILES

Thermophiles are heat-loving organisms and can be either bacteria or molds. Thermophilic and hyperthermophilic aquatic microorganisms (see 8.1.1 Archaeans) require unique environmental and nutritional conditions to survive (e.g., presence of specific inorganic or organic nutrients and their concentrations, extreme pH, presence or absence of oxygen). These conditions do not exist in the high-purity water of pharmaceutical water systems, whether ambient or hot, to support their growth. Bacteria that are able to inhabit hot pharmaceutical water systems are invariably found in much cooler locations within these hot systems; for example, within infrequently used outlets, ambient subloops off of hot loops, use-point and sub-loop cooling heat exchangers, transfer hoses and connecting pipes, or dead legs. These bacterial contaminants are the same mesophilic (moderate temperature-loving) types found in ambient water systems and are not thermophiles. Based on these facts, thermophilic bacteria are not a concern for hot pharmaceutical-grade water systems.

### 8.2 Biofilm Formation in Water Systems

A biofilm is a three-dimensional structured community of sessile microbial cells embedded in a matrix of extracellular polymeric substances (EPS). Biofilms form when bacteria attach to surfaces in moist environments and produce a slimy, glue-like substance, the EPS matrix, while proliferating at that location. This slimy matrix facilitates biofilm adhesion to surfaces as well as the attachment of additional planktonic cells to form a microbial community.

The EPS matrix of biofilms that colonize water systems also facilitates adsorption and concentration of nutrients from the water and retains the metabolites and waste products produced by the embedded biofilm cells, which can serve as nutrients for other biofilm community members.

This EPS matrix is also largely responsible for biofilm’s resistance to chemical sanitizers, which must penetrate completely through the matrix to contact and kill the biofilm cells within the matrix. Heat sanitization approaches do not generally have
these EPS matrix penetration difficulties, so they are usually considered superior to chemicals in killing biofilms where materials of construction allow.

The three-dimensional structure of a well-developed biofilm, as well as the biofilm’s creation and release of small, motile “pioneer cells” for further colonization, are facilitated through gene expression modulating “quorum sensing” chemicals released in tiny amounts by individual biofilm cells and concentrated to a functional level within this same EPS matrix. So, the EPS matrix of biofilms is primarily responsible for the biofilm’s success in colonizing and proliferating in very low nutrient-containing high-purity water systems. The EPS matrix also explains the difficulty in killing and/or removing biofilms from water purification and distribution system surfaces.

### 8.2.1 BIOFILM-FORMING BACTERIA IN WATER SYSTEMS

Common microorganisms recovered from water system samples include Gram-negative bacteria from the genera 
Pseudomonas, Ralstonia, Burkholderia, Stenotrophomonas, Comamonas, Methylobacterium,
and many other types of 
Pseudomonas-like organisms known collectively as pseudomonads (*members of the family Pseudomonadaceae*). These types of microbes, found in soil and source water, tend to colonize all water system distribution and purification system surfaces including activated carbon beds, deionizing resin beds, RO systems, membrane filtration modules, connecting piping, hoses, and valves. If not controlled, they can compromise the functionality of purification steps in the system and spread downstream, possibly forming biofilms on the distribution system surfaces such as tanks, piping, valves, hoses, and other surfaces, from where they can be sheared or otherwise released into the finished water used in processes and products.

Some of the biofilm pseudomonads are opportunistic human pathogens and may possess resistance to commonly used pharmaceutical product preservatives, particularly when embedded in EPS matrix flocs sheared from water system biofilms. Several pseudomonads are also capable of utilizing a wide variety of carbon sources as nutrients, allowing them to colonize austere, adventitious nutrient environments such as water systems. This nutritional diversity also makes them capable of growing to very high numbers in some pharmaceutical products and raw materials, thus leading to product adulteration and potential risk to patient health. Given that these bacteria are commonly found in aqueous environments, endotoxin control for Water for Injection systems (and some Purified Water systems) through biofilm control becomes critical.

### 8.2.2 NON-BIOFILM-FORMING BACTERIA IN WATER SYSTEMS

Other types of non-pseudomonad Gram-negative bacteria, such as the genera Escherichia, Salmonella, Shigella, Serratia, Proteus, Enterobacter, and Klebsiella, are used as indicators of fecal contamination. Although some of these bacteria are also plant pathogens, others can be human enteric pathogens, and can contaminate potable water supplies. These non-pseudomonads are not suited to colonizing or surviving in pharmaceutical water systems unless local sewage and source water controls are not in place. Such controls are required in order to comply with the source water requirements for making USP-grade waters as described in their respective monographs.

### 8.3 Microorganism Sources

#### 8.3.1 EXOGENOUS CONTAMINATION

Exogenous microbial contamination of bulk pharmaceutical water comes from numerous possible sources, including source water. At a minimum, source water should meet the microbial quality attributes of Drinking Water, which is the absence of fecal coliforms (*E. coli*). A wide variety of other types of microorganisms, chiefly Gram-negative bacteria, may be present in the incoming water. If appropriate steps are not taken to reduce their numbers or eliminate them, these microorganisms may compromise subsequent water purification steps.

Exogenous microbial contamination can also arise from maintenance operations, equipment design, and the process of monitoring, including:

- Unprotected, faulty, or absent vent filters or rupture disks
- Backflow from interconnected equipment
- Non-sanitized distribution system openings for component replacements, inspections, repairs, and expansions
- Inadequate drain air-breaks
- Innate bioburden of activated carbon, ion-exchange resins, regenerant chemicals, and chlorine-neutralizing chemicals
- Inappropriate rinsing water quality after regeneration or sanitization
- Poor sanitization of use points, hard-piped equipment connectors, and other water transfer devices such as hoses
- Deficient techniques for use, sampling, and operation

The exogenous contaminants may not be normal aquatic bacteria but rather microorganisms of soil, air, or even human origin. The detection of non-aqueous microorganisms may be an indication of sampling or testing contamination or a system component failure, which should trigger investigation and remediation. Sufficient care should be given to sampling, testing, system design, and maintenance to minimize microbial contamination from exogenous sources.

#### 8.3.2 ENDOGENOUS CONTAMINATION

Endogenous sources of microbial contamination can arise from unit operations in a water purification system that is not properly maintained and operated. Microorganisms present in source water may adsorb to carbon bed media, ion-exchange resins, filter membranes, and other equipment surfaces, and initiate the formation of biofilms.
Downstream colonization can occur when microorganisms are shed from existing biofilm-colonized surfaces and carried to other areas of the water system. Microorganisms may also attach to suspended particles such as carbon bed fines or fractured resin particles. When the microorganisms become planktonic, they serve as a source of contamination to subsequent purification equipment and to distribution systems.

Another source of endogenous microbial contamination is the distribution system itself. Microorganisms can colonize pipe surfaces, rough welds, misaligned flanges, valves, and dead legs, where they proliferate and form biofilms. Once formed, biofilms can become a continuous source of microbial contamination, which is very difficult to eradicate. Therefore, biofilm development must be managed by methods such as frequent cleaning and sanitization, as well as process and equipment design.

8.4 Endotoxin

Endotoxin remediation may be accomplished through the normal exclusion or removal action afforded by various unit operations within the treatment system. Examples of endotoxin removal steps in a water purification train include RO, deionization, ultrafilters, distillation, and endotoxin-adsorptive filters.

8.4.1 SOURCES

Endotoxins may be introduced into the system from the source water or may be released from cell surfaces of bacteria in water system biofilms. For example, a spike in endotoxin may occur following sanitization as a result of endotoxin release from killed cells. Endotoxin quantitation in water samples is not a good indicator of the level of biofilm development in a water system because of the multiplicity of endotoxin sources.

8.4.2 REMOVAL AND CONTROL

To control endotoxin levels in water systems, it is important to control all potential sources of contamination with Gram-negative bacteria as well as free endotoxin in the water. Contamination control includes the use of upstream unit operations to reduce bioburden from incoming water, as well as engineering controls (e.g., heat sanitization, equipment design, UV sanitizers, filters, material surface, and flow velocity) to minimize biofilm development on piping surfaces and to reduce re-inoculation of the system with free-floating bacteria.

Endotoxin remediation may be accomplished through the normal exclusion or removal action afforded by various unit operations within the treatment system. Examples of endotoxin removal steps in a water purification train include RO, deionization, ultrafilters, distillation, and endotoxin-adsorptive filters.

8.5 Test Methods

Microbes in water systems can be detected as exampled in this section or by methods adapted from Microbial Enumeration Tests (61), Tests for Specified Microorganisms (62), or the current edition of Standard Methods for the Examination of Water and Wastewater by the American Public Health Association. This section describes classical culture approaches to bioburden testing, with a brief discussion on rapid microbiological methods.

Every water system has a unique microbiome. It is the user’s responsibility to perform method validation studies to demonstrate the suitability of the chosen test media and incubation conditions for bioburden recovery. In general, users should select the method that recovers the highest planktonic microbial counts in the shortest time, thus allowing for timely investigations and remediation. Such studies are usually performed before or during system validation.

The steady state condition can take months or even years to be achieved, and can be affected by a change in source water quality, changes in finished water purity by using modified or increasingly inefficient purification processes, changes in finished water use patterns and volumes, changes in routine and preventative maintenance or sanitization procedures and frequencies, or any type of system intrusion (e.g., component replacement, removal, or addition).

8.5.1 MICROBIAL ENUMERATION CONSIDERATIONS

Most microbial contaminants in water systems are found primarily as biofilms on surfaces, with only a very small percentage of the microbiome suspended in the water, or planktonic, at any given time. Although it would seem logical to directly monitor biofilm development on surfaces, current technology for surface evaluations in an operating water system makes this impractical in a GMP environment. Therefore, a indirect approach must be used: the detection and enumeration of planktonic microorganisms that have been released from biofilms. This planktonic microbiome will impact the processes or products where the water is used.

The detection and enumeration of the planktonic microbiome can be accomplished by collecting samples from water system outlets. Planktonic organisms are associated with the presence of biofilms as well as free-floating bacteria introduced into the system (pioneer cells), which may eventually form new biofilms. Therefore, by enumerating the microorganisms in water samples, the overall state of control over biofilm development can be assessed. This assessment has historically been accomplished with classical cultural techniques, which are viewed as the traditional method. However, nutritional limitations of the growth media may not satisfy growth requirements of organisms present in the water system that originated from a...
biofilm. As a result, traditional cultural methods may only detect a fraction of the biofilm bacteria present in the water sample. Other options are available, such as rapid microbiological methods.

There is no ideal cultural enumeration method that will detect all microorganisms in a water sample, although some media or incubation temperatures may be better than others. However, from a PC perspective, this limitation is acceptable because it is the relative changes in the trends for water sample microbial counts that indicate the state of PC.

▲ Consideration should also be given to the timeliness of microbial testing after sample collection. The number of detectable organisms in a sample collected in a sterile, scrupulously clean sample container will usually decrease as time passes. The organisms within the sample may die or adhere to the container walls, reducing the number that can be withdrawn from the sample for testing. The opposite effect can also occur if the sample container is not scrupulously clean and contains a low concentration of nutrients that could promote microbial growth. Because the number of organisms in the water can change over time after sample collection, it is best to test the samples as soon as possible. If it is not possible to test the sample within 2 h of collection, the sample should be held at refrigerated temperatures (2°–8°) and tested within 24 h. In situations where even 24 h is not possible (such as when using off-site contract laboratories), it is particularly important to qualify the microbiological sample hold times and storage conditions to avoid significant changes in the microbial population during sample storage. ▲ 1S (USP41)

8.5.2 THE CLASSICAL CULTURAL APPROACH

Classical cultural approaches for microbial testing of water include but are not limited to pour plates, spread plates, membrane filtration, and most probable number (MPN) tests. These methods are generally easy to perform, and provide excellent sample processing throughput. Method sensitivity can be increased via the use of larger sample sizes. This strategy is used in the membrane filtration method. Cultural approaches are further defined by the type of medium used in combination with the incubation temperature and duration. This combination should be selected according to the monitoring needs of a specific water system and its ability to recover the microorganisms of interest, i.e., those that could have a detrimental effect on the products manufactured or process uses, as well as those that reflect the microbial control status of the system.

8.5.2.1 Growth media: The traditional categorization is that there are two basic forms of media available: “high nutrient” and “low nutrient”. Those media traditionally categorized as high-nutrient include Plate Count Agar (TGYA), Soybean Casein Digest Agar (SCDA or TSA), and m-HPC Agar (formerly m-SPC Agar). These media are intended for the general isolation and enumeration of heterotrophic or copiotrophic bacteria. Low-nutrient media, such as R2A Agar and NWRI Agar (HPCA), have a larger variety of nutrients than the high-nutrient media. These low-nutrient media were developed for use with potable water due to their ability to recover a more nutritionally diverse population of microorganisms found in these environments. The use of R2A may not be the best choice for high-purity water systems. Even though high-purity water creates an oligotrophic environment, it has been shown empirically that in many high-purity compendial waters, the microbial count disparity between low- and high-nutrient media is dramatically less to nil, compared to potable water. Nevertheless, using the medium that has been demonstrated ▲ as acceptable through comparative media analysis is recommended. ▲ 1S (USP41)

8.5.2.2 Incubation conditions: Duration and temperature of incubation are also critical aspects of microbiological testing. Classical compendial methods (e.g., (61)) specify the use of high-nutrient media, typically incubated at 30°–35° for NLT 48 h. Given the types of microbes found in many water systems, incubation at lower temperatures (e.g., ranges of 20°–25° or 25°–30°) for longer periods (at least 4 days) could recover higher microbial counts than classical compendial methods. Low-nutrient media typically require longer incubation conditions (at least 5 days) because the lower nutrient concentrations promote slower growth. Even high-nutrient media can sometimes yield higher microbial recovery with longer and cooler incubation conditions.

8.5.2.3 Selection of method conditions: The decision to test a particular system using high- or low-nutrient media, higher or lower incubation temperatures, and longer or shorter incubation times should be based on comparative cultivation studies using the native microbiome of the water system. The decision to use media requiring longer incubation periods to recover higher counts also should be balanced with the timeline of results. Detection of marginally higher counts at the expense of a significantly longer incubation period may not be the best approach for monitoring water systems, particularly when the slow growers are not new species but the same as those recovered within shorter incubation times. Some cultural conditions using low-nutrient media lead to the development of microbial colonies that are much less differentiated in colonial appearance, an attribute that microbiologists rely on when selecting representative microbial types for further characterization. The nature of some of the slow growers and the extended incubation times needed for their development into visible colonies also may lead to those colonies becoming dysgonic and difficult to subculture. That could limit their further characterization, depending on the microbial identification technology used. The selection of method parameters should provide conditions that adequately recover microorganisms from the water system, including those that are objectionable for the intended water use. ▲ 1S (USP41)

8.5.3 SUGGESTED CLASSICAL CULTURAL METHODS

▲ Example methods are presented in Table 3. ▲ 1S (USP41)

<table>
<thead>
<tr>
<th>Table 3: 1S (USP41) Example Culture Methods</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Drinking Water</strong></td>
</tr>
<tr>
<td>Pour plate method or membrane filtration method a</td>
</tr>
<tr>
<td>Suggested sample volume: 1.0 mL b</td>
</tr>
<tr>
<td>Growth medium: ▲ Plate Count Agar ▲ 1S (USP41) c</td>
</tr>
<tr>
<td>Incubation time: 48–72 h d</td>
</tr>
<tr>
<td>Incubation temperature: 30°–35° e</td>
</tr>
</tbody>
</table>

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### 8.5.4 MICROBIAL IDENTIFICATION

In addition to the enumeration of the bioburden in the water, there is a need to identify and/or select certain microbial species that could be detrimental to products or processes. Some bacteria may also be resistant to preservatives and other antimicrobial chemicals used in nonsterile liquid and semi-solid products, thus leading to potential product spoilage. For example, *Pseudomonas aeruginosa* and *Burkholderia cepacia*, as well as some other pseudomonads, are known opportunistic pathogens under certain conditions. As such, it may be appropriate to consider these species as objectionable microorganisms for the type of water used to manufacture nonsterile liquid and semi-solid products. There is a higher risk of infection if these organisms are found in products targeted for susceptible patient populations (e.g., the very young, the very old, and the immunocompromised) or products contacting highly susceptible tissues (e.g., inhaled products or some topical products). However, if the product where the water is used carries an absence specification for a particular pathogenic species that is not capable of living in a high-purity water system (e.g., *Staphylococcus aureus* or *Escherichia coli*), then these non-aquatic species should not be candidates for routine recovery testing from water samples. For more information, see *Microbiological Examination of Nonsterile Products: Acceptance Criteria for Pharmaceutical Preparations and Substances for Pharmaceutical Use* (1111), *Microbial Characterization, Identification, and Strain Typing* (1113), and *Microbiological Best Laboratory Practices* (1117).

For PC and QC, it is valuable to know the microbial species present in the normal microbiome of a water system, even if they are not specifically objectionable. If a new species is detected, it may be an indication of a subtle process change or an exogenous intrusion. The identity of the microorganism may be a clue as to its origin and can help with implementation of corrective or preventive action. Therefore, it is industry practice to identify the microorganisms in samples that yield results exceeding established Alert and Action Levels. It is also of value to periodically identify the normal microbiome in a water system, even if they are below established Alert Levels. This information can provide perspective on the species recoveries from Alert and Action Level excursion samples, indicating whether they are new species or just higher levels of the normal microbiome. Water system isolates may be incorporated into a company culture collection for use in tests such as antimicrobial effectiveness tests, microbial method validation/suitability testing, and media growth promotion. The decision to use water isolates in these studies should be risk-based because many such isolates may not grow well on the high-nutrient media required. And because once adapted to laboratory media, they may not perform like their wild type progenitors.

### 8.5.5 RAPID MICROBIOLOGICAL METHODS

In recent years, new technologies that enhance microbial detection and the timeliness of test results have been adopted by pharmaceutical QC testing labs. Rapid Microbiological Methods (RMM) are divided into four categories: Growth-Based, Viability-Based, Metabolite-Based, and Nucleic Acid-Based. Examples of RMM used for the evaluation of microbial quality of water systems include:

- Microscopic visual epifluorescence membrane counting techniques
- Automated laser scanning membrane counting approaches
- Early colony detection methods based on autofluorescence, adenosine triphosphate (ATP) bioluminescence, or vital staining
- Genetic-based detection/quantitation

See *Validation of Alternative Microbiological Methods* (1223) for further information on rapid microbiological methods.
6.1 Purposes and Procedures

for making decisions regarding the immediate processing and usability of the water (see the water intended by the purpose of the sampling, i.e., for PC or for QC. sample collection. Physical attributes such as the pressure drop across a filter, temperature, and flow rate—which are sometimes considered critical for operation or sanitization of the water system—must be measured in situ during operation. Obtaining timely microbial data is more challenging compared to chemical and physical attributes, often taking several days. This limits the ability to control microbial attributes in a timely manner, and therefore requires a more challenging evaluation of the test results and conservative implementation of PC levels. This section provides guidance on the establishment and use of Alert and Action Levels, as well as Specifications to assess the suitability of the water and the water system for use in production.

9. ALERT AND ACTION LEVELS AND SPECIFICATIONS

9.1 Introduction

Establishment of Alert and Action Levels for any manufacturing process facilitates appropriate and timely control. In the case of a pharmaceutical water system, the key PC parameters can be specific chemical, physical, and microbiological attributes of the water produced. Typically, most chemical attributes can be determined in real time or in the lab within a few minutes after sample collection. Physical attributes such as the pressure drop across a filter, temperature, and flow rate—which are sometimes considered critical for operation or sanitization of the water system—must be measured in situ during operation. Obtaining timely microbial data is more challenging compared to chemical and physical attributes, often taking several days. This limits the ability to control microbial attributes in a timely manner, and therefore requires a more challenging evaluation of the test results and conservative implementation of PC levels. This section provides guidance on the establishment and use of Alert and Action Levels, as well as Specifications to assess the suitability of the water and the water system for use in production.

9.2 Examples of Critical Parameter Measurements

Examples of measurements and parameters that are important to water system processes and products are described below. The list, which is not intended to be exhaustive or required, contains some examples of parameters that could be measured to demonstrate that the system is in a state of control.

Examples of measurements that could be critical to the purification or sanitization process include:

- Temperature, for thermally sanitized systems
- Percent rejection of an RO system
- Endotoxin levels of feed water to a distillation system
- Chlorine presence immediately prior to an RO system

Examples of measurements that could be critical to the water distribution process include:

- Return/end-of-loop line pressure, to forewarn of the potential to aspirate air or fluids because of simultaneous use of too many outlets
- Temperature to assure the self-sanitizing conditions are maintained for a hot water system
- Flow rate, to ensure that sufficient water is available for operations

Examples of measurements that could be critical to final water quality include:

- Conductivity
- TOC
- Endotoxin—for Water for Injection systems
- Bioburden
- Ozone or other chemicals—for chemically sanitized systems

9.3 Purpose of the Measurements

Although the purpose of each measurement varies, the results can be used to provide system performance feedback, often immediately, serving as ongoing PC and product quality indicators. At the same time, the results provide information necessary for making decisions regarding the immediate processing and usability of the water (see 6.1 Purposes and Procedures). However, some attributes may not be monitored continuously or may have a long delay in data availability (e.g., microbial data). Regardless, both real-time data and data with longer cycle times can be used to properly establish Alert and Action Levels, which can serve as an early warning or indication of a potentially approaching quality shift.

As PC indicators, Alert and Action Levels are trigger points for the potential need for investigation and/or remedial action, to prevent a system from deviating from normal conditions and producing water unsuitable for its intended use. This “intended use” minimum quality is sometimes referred to as a “Specification” or “Limit”, and may include limits for conductivity and TOC listed in water monographs, or other specifications required for these waters that have been defined by the user internally.

In all cases, the validity of the data should be verified to ensure that the data are accurate and consistently representative of the water quality in the system, regardless of whether the sample was collected from a sampling point or use point. The resulting data must not be unduly biased, positively or negatively, due to the sampling method, the environment in the vicinity of the sampling location, the test procedure, instrumentation, or other artifacts that could obscure or misrepresent the true quality of the water intended by the purpose of the sampling, i.e., for PC or for QC.

9.4 Defining Alert and Action Levels and Specifications

Data generated from routine water system monitoring should be trended to ensure that the system operates in a state of chemical and microbiological control. To assist with the evaluation of system performance, companies should establish in-process control levels based on historical data or a fraction of the water Specifications (as long as this latter approach yields values with relevance to process performance).

When establishing Alert and Action Levels and Specifications, a two- or three-tier approach is typically used. In a three-tier approach, the typical structure is to establish in-process controls using “Alert Level”, “Action Level”, and “Specifications”. Alert
and Action Levels are used as proactive approaches to system management prior to exceeding Specifications. The criteria for defining and reacting to adverse trends should be set by the user. These levels should be set at values that allow companies to take action to prevent the system from producing water that is unfit for use. Water Specifications or Limits represent the suitability for use of the water.

In a two-tier approach, a combination of the above terminology is used, depending on the parameter to be monitored. For example, if the attribute does have a monograph specification, the two tiers are Alert Level (or Action Level) and Specification. If the attribute does not have a limit/specification, the two tiers are usually Alert Level and Action Level.

A single-tier approach is possible, but this is risky and difficult to manage. With this approach, where the water/system is either acceptable or not acceptable, the single-tier method does not allow for any adjustment, correction, or investigation prior to stopping production.

However, certain sampling locations, such as sampling ports that are not used for manufacturing products or processes, do not represent the finished water quality where a Specification could be applied. In these locations, a two-tier approach (Alert and Action Levels only) could be applied. In some sampling locations, a single PC level might possibly be appropriate, depending on the attribute.

9.4.1 ALERT LEVEL

An Alert Level for a measurement or parameter should be derived from the normal operating range of the water system. Specifically, Alert Levels are based on the historical operating performance under production conditions, and then are established at levels that are just beyond the majority of the normal historical data. The Alert Level for a parameter is often a single value or a range of values, such as:

- Higher than typical conductivity or TOC
- Higher than typical microbial count
- Higher than typical endotoxin level
- Low temperature during thermal sanitization
- pH range control prior to an RO
- Ozone concentration in a storage tank

Various methods, tools, and statistical approaches are available for establishing Alert Levels, and the user needs to determine the approaches that work for their application. Some numerical examples are two or three standard deviations \( \sigma \) (or more) in excess of the mean value, or some percentage above the mean value but below a Specification. An event-based example could be the appearance of a new microorganism or a non-zero microbial count where zero is the norm.

When an Alert Level is exceeded, this indicates that a process or product may have drifted from its normal operating condition or range. Alert Level excursions represent a warning and do not necessarily require a corrective action. However, Alert Level excursions may warrant notification of personnel involved in water system operation, as well as the quality assurance (QA) personnel. Alert Level excursions may also lead to additional monitoring, with more intense scrutiny of the resulting and neighboring data as well as other process indicators.

9.4.2 ACTION LEVEL

An Action Level is also based on the same historical data, but the levels are established at values (or ranges) that exceed the Alert Levels. The values/ranges are determined using the same types of numerical or event-based tools as the Alert Levels, but at different values\( \sigma \)/ranges.\( ^{15} \) (USP21)

In a three-tier approach, it is good practice to select an Action Level that is more than the Alert Level, but less than the Specification to allow the user to make corrective actions before the water would go out of compliance.

Exceeding a quantitative Action Level indicates that the process has allowed the product quality or other critical parameter to drift outside of its normal operating range. An Action Level can also be event-based. In addition to exceeding quantitative Action Levels, some examples of event-based Action Level excursions include, but are not limited to:

- Exceeding an Alert Level repeatedly
- Exceeding an Alert Level in multiple locations simultaneously
- The recovery of specific objectionable microorganisms
- A repeating non-zero microbial count where zero is the norm

If an Action Level is exceeded, this should prompt immediate notification of both QA staff and the personnel involved in water system operations and use, so that corrective actions can be taken to restore the system back to its normal operating range. Such remedial actions should also include investigative efforts to understand what happened and eliminate or reduce the probability of recurrence. Depending on the nature of the Action Level excursion, it may be necessary to evaluate its impact on the water uses during the period between the previous acceptable test result and the next acceptable test result.

9.4.3 SPECIAL ALERT AND ACTION LEVEL SITUATIONS

In new or significantly altered water systems, where there is limited or no historical data from which to derive trends, it is common to establish initial Alert and Action Levels based on equipment design capabilities. These initial levels should be within the process and product Specifications where water is used. It is also common for new water systems, especially ambient water systems, to undergo changes, both chemically and microbiologically, over time as various unit operations (such as RO membranes) exhibit the effects of aging. This type of system aging effect is most common during the first year of use. As the system ages, a steady state \( \mu \text{microbiome} \) (microorganism types and levels) may develop due to the collective effects of system design, source water, maintenance, and operation, including the frequency of re-bedding, backwashing, regeneration, and sanitization. This established or mature \( \mu \text{microbiome} \) may be higher than the one detected when...
the water system was new. Therefore, there is cause for the impurity levels to increase over this maturation period and eventually stabilize.

Some water systems are so well controlled microbially—such as continuously or intermittently hot Water for Injection distribution systems—that microbial counts and endotoxin levels are essentially nil or below the limit of reasonable detectability. This common scenario often coincides with a very low Specification that is poorly quantifiable due to imprecision (as much as two-fold variability) of the test methods that may be near their limits of detection. In such systems, quantitative data trending has little value, and therefore, quantitative PC levels also have little value. The non-zero values in such systems could be due to sporadic sampling issues and not indicative of a water system PC deviation; however, if these non-zero values occur repeatedly, they could be indicative of process problems. So, an alternative approach for establishing Alert and Action Levels with these data could be the use of the incident rate of non-zero values, with the occasional single non-zero “hit” perhaps being an Alert Level (regardless of its quantitative value), and multiple or sequential “hits” being an Action Level. Depending on the attribute, perhaps single hits may not even warrant being considered an Alert Level, so only a multiple-hit situation would be considered actionable. It is up to the user to decide on their approach for system control, i.e., whether to use one, two, or three levels of controls for a given water system and sampling location, and whether to establish Alert and Action Levels as quantitative or qualitative hit-frequency values.

9.4.4 SPECIFICATIONS

Water Specifications or Limits are set based on direct potential product and/or process impact and they represent the suitability for use of the water. The various bulk water monographs contain tests for Conductivity, TOC, and Bacterial Endotoxins (for Water for Injection). Aside from the monographs for Water for Hemodialysis and multiple sterile waters, microbial specifications for the bulk waters are intentionally not included in their monograph tests. The need for microbial specifications for bulk waters (Purified Water and Water for Injection) depends on the water use(s), some of which may require strict control (e.g., very low bioburden, absence of objectionable organisms, or low ionic strength) while others may require no specification due to the lack of impact. For example, microbial specifications are appropriate and typically expected for water that is used in product formulations and final equipment rinses. *Where the water is used for analytical reagent preparations and the analytical method is not affected by microbial contaminants* or for cleaning processes that conclude with a final antimicrobial heat drying or solvent rinsing step, the microbial quality of the water is likely less of a concern. The decision to establish microbial Specifications for bulk pharmaceutical waters should be based on a formal risk assessment of its uses and justified by scientific rationale.

It is very important to understand the chemical and microbial quality of the water in its final form as it is delivered from a water system to the locations where it is used in manufacturing activities and other points of use. The quality of the water within the water system could be compromised if it picks up chemical or microbial contaminants during its delivery from the system to the points of use. These points of use, where cumulative contamination could be present, are the locations where compliance with all the water Specifications is mandated.

As discussed above, compliance with chemical Specifications can be confirmed periodically between uses, immediately prior to use, or even while the water is being utilized in product manufacturing. While the use of RMM may provide for timely microbial data, the use of conventional cultiative microbiological testing usually delays confirmation of microbial compliance until after the water has been used. However, for some applications, this logistical limitation should not eliminate the need for establishing microbial Specifications for this very important raw material.

The manufacturing risk imposed by these logistics accentuates the value of validated microbial control for a water system. It also emphasizes the value of *unbiased sampling for microbial monitoring* (e.g., influences from technique, hoses, flushing) of samples collected from pertinent locations, with evaluation of the resulting data against well-chosen, preferably trend-derived Alert and Action Levels, which can facilitate remedial PC to preclude Specification excursions.

Users should establish their own quantitative microbial Specifications suited to their water uses. But these values should not be greater than 100 cfu/mL for Purified Water or 10 cfu/100 mL for Water for Injection unless specifically justified, because these values generally represent the highest microbial levels for pharmaceutical water that are still suitable for manufacturing use.

A Specification excursion should prompt an out-of-specification (OOS) investigation. The investigation is performed to determine 1) the root cause of the excursion so that CAPA may be taken for remediation purposes, and 2) assess the impact on affected processes and finished products where the water was used. Product disposition decisions must be made and are dependent on factors that could include:

- Role of water in the product or in-process material
- Chemical or microbial nature of the attribute whose Specification value was exceeded
- Level of product contamination by the water
- Presence of objectionable microorganisms
- Any downstream processing of affected in-process materials that could mitigate the OOS attribute
- Physical and chemical properties of the finished product where the water was used that could mitigate the OOS attribute
- Product administration routes and potentially sensitive/susceptible users

9.4.5 SOURCE WATER CONTROL

The chemical and microbial attributes of the starting source water are important to the ability of the water system to remove or reduce these impurities to meet the finished water Specifications (see 2. Source Water Considerations). Using the example microbial enumeration methods in Table 3 of Water for Injection, a reasonable maximum bacterial Action Level for source water is 500 cfu/ML. This number is derived from U.S. EPA NPDWR where it is used as an Action Level for the water authority indicating the need for improving disinfection and water filtration to avoid the penetration of viral, bacterial, and protozoal pathogens into
the finished Drinking Water. It is not, however, a U.S. EPA heterotrophic plate count Specification or Maximum Contaminant Level (MCL) for Drinking Water.

Nevertheless, of particular importance could be the microbial and chemical quality of this starting water because the water is often delivered to the facility at a great distance from its source and in a condition over which the user has little or no control. High microbial and chemical levels in source water may indicate a municipal potable water system upset, a change in the supply or original water source, a broken water main, or inadequate disinfection, and therefore, potentially contaminated water with objectionable or new microorganisms or coincidental chemical contaminants.

Considering the potential concern about objectionable microorganisms and chemical contaminants in the source water, contacting the water provider about the problem should be an immediate first step. In-house remedial actions could also be needed, including performance of additional testing on the incoming water (as well as the finished water in some cases) or pretreating the water with additional microbial and chemical purification operations (see 5.1 Unit Operations Considerations).

1235 VACCINES FOR HUMAN USE—GENERAL CONSIDERATIONS

INTRODUCTION

Vaccines have been used for centuries to immunize individuals against pathogenic organisms with the goal of preventing the associated disease. Vaccines are biological products that contain antigens capable of inducing a specific and active acquired immune response in the body. Antigens present in vaccines are processed by specialized cells in the body’s immune system, resulting in the development of blood proteins known as antibodies (i.e., humoral immunity) or specialized lymphocytes (i.e., cell-mediated immunity) or both. Therefore immune responses may be antibody mediated, cell mediated, or both. Thus, antigens are critical for vaccine function and generally consist of a portion of the pathogenic organism, or an attenuated form of the whole microorganism. In the case of DNA-based vaccines (currently under development), the vaccine would contain nucleotide sequences (genetic material) that encode microbial antigens.

Examples of types of licensed vaccines appear in Appendix 1. A current list of vaccines licensed in the United States is posted at www.fda.gov/cber/.

Vaccines can be of various types, depending on their design and processes involved in their manufacture. Vaccines for human use may contain whole killed or attenuated organisms (e.g., bacteria or viruses) or contain antigens derived from portions of a pathogen, either by partitioning and purification or derived using recombinant technology (Table 1). Some polysaccharide vaccines are conjugated to a carrier in order to enhance their immune response.

<table>
<thead>
<tr>
<th>Table 1. Bacterial and Viral Vaccines</th>
</tr>
</thead>
<tbody>
<tr>
<td>Live attenuated whole cell or virus*</td>
</tr>
<tr>
<td>Inactivated/killed*</td>
</tr>
<tr>
<td>Whole cell or virus*</td>
</tr>
<tr>
<td>Recombinant proteins*</td>
</tr>
<tr>
<td>Subunit*</td>
</tr>
<tr>
<td>Polysaccharides</td>
</tr>
<tr>
<td>Proteins</td>
</tr>
<tr>
<td>Modified toxins</td>
</tr>
</tbody>
</table>

* Live attenuated bacterial or viral vaccines are weakened (attenuated) forms of a pathogen. They contain antigens that are similar to disease-causing microbes. They may be derived from the pathogen itself, or from a different organism that contains antigens that cross-react with the virulent microbe (e.g., vaccinia and variola).

** Inactivated bacterial and viral vaccines are produced by growing cells of disease-causing bacteria or viruses in cell substrates and subsequently inactivating them to prevent replication in the recipient.

† Inactivated/killed whole-cell or virus vaccines consist of the entire microorganisms after they have been inactivated. These preparations may or may not be partially or completely purified.

‡ Recombinant protein viral and bacterial vaccines are derived from host cells that have been transformed with expression vectors that carry genes that encode antigenic material from infectious agents. The expression cells are grown in bioreactors to produce the recombinant antigenic material.

§ Subunit vaccines are extracts from inactivated/killed viruses or bacteria. Subunit-type vaccines generally undergo some degree of purification.

In addition to antigen(s), vaccines may contain several other components, such as adjuvants that enhance the immune response to the vaccine antigen, preservatives to prevent bacterial or fungal contamination of multiple-dose vials, or other excipients needed for pharmaceutical manufacturing or vaccine stabilization. Residual components from the manufacturing process also may be present in vaccine preparations. Examples of these categories are listed in Table 2.

<table>
<thead>
<tr>
<th>Table 2. Vaccine Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antigens</td>
</tr>
<tr>
<td>Whole organisms</td>
</tr>
<tr>
<td>Components/subunits</td>
</tr>
<tr>
<td>Recombinant proteins</td>
</tr>
</tbody>
</table>
Table 2. Vaccine Components (continued)

<table>
<thead>
<tr>
<th>Adjuvants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aluminum salts</td>
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<table>
<thead>
<tr>
<th>Antimicrobial preservatives</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thimerosal</td>
</tr>
<tr>
<td>2-Phenoxyethanol</td>
</tr>
<tr>
<td>Benethonium chloride</td>
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<tr>
<td>Phenol</td>
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</tbody>
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<table>
<thead>
<tr>
<th>Stabilizers</th>
</tr>
</thead>
<tbody>
<tr>
<td>salts</td>
</tr>
<tr>
<td>amino acids</td>
</tr>
<tr>
<td>sugars</td>
</tr>
<tr>
<td>proteins</td>
</tr>
<tr>
<td>other</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Manufacturing residuals</th>
</tr>
</thead>
<tbody>
<tr>
<td>cell-derived residuals</td>
</tr>
<tr>
<td>materials of animal origin</td>
</tr>
<tr>
<td>antibiotic residuals</td>
</tr>
<tr>
<td>inactivating chemical agents</td>
</tr>
<tr>
<td>other</td>
</tr>
</tbody>
</table>

Different vaccine antigens are often combined in one final formulation in order to elicit immunity against multiple diseases and to reduce the number of separate administrations needed to achieve immunity to the various vaccine antigens. Despite the multiple forms vaccines may take, several common features characterize the manufacture and testing of vaccines. This chapter focuses on commonalities throughout the manufacturing process, from raw material qualifications to final release tests.

Regulations and Standards

Vaccines are regulated by FDA as biological products. The general requirements are listed in national laws and international guidances. For the U.S., national requirements are codified in 21 CFR, the 200 and 600 sections, with additional recommendations available in FDA Points to Consider and Guidance documents (www.fda.gov). International guidances are available from the International Conference on Harmonization (ICH) (www.ich.org; see Appendix 2) and the World Health Organization. New methodologies are continually being developed and validated and will be included in USP as they become available. Reference standards are available from USP and FDA.

OVERALL MANUFACTURING PLAN

When considering the overall plan for manufacturing a vaccine, manufacturers need to consider the following factors:

- Physical facilities;
- Raw materials and process aids;
- Actual manufacturing process, including
  1. Initial process (production of virus/bacteria and recombinant materials);
  2. Downstream processes (purification or chemical modification, if applicable);
- Antigen modifications such as conjugation or toxoiding;
- Storage of process intermediates and final bulk;
- In-process and final product testing regimens and control schemes;
- Addition of adjuvants, if applicable;
- Formulation and filling;
- Container–closure system; and
- Stability program that supports the dating period of the product.

Quality systems are needed to support the following manufacturing process development: specifications for raw materials, process intermediates, and final product; change control; and failure investigations and complaints. All of these elements are important in the life cycle of the vaccine product.

The overall goal of a comprehensive manufacturing program is to consistently produce a vaccine that is safe and effective. Concurrently with clinical development of the vaccine, the manufacturing process is refined and the process and testing methods are validated for consistency. This includes systems to control changes to the process or inputs. Manufacturers should expect that changes will be required during the vaccine’s manufacturing life cycle, and manufacturers necessarily will use data to support these changes.
from development and routine manufacturing to assess the process as well as proposed changes. The manufacturers should adopt systems that continually evaluate all aspects of manufacturing to identify unanticipated changes in vaccine quality and to assess them as quickly as possible.

Manufacturing Facilities and Systems

Manufacturers should have a general layout of manufacturing facilities, including diagrams that show the following: flow of raw materials and process inputs; movement of product, intermediates, waste streams, and personnel; and air flows and pressurization levels. These diagrams assist in minimizing the risk of potential product contamination from various sources. These sources can include cross-contamination from other products, contamination from different batches of the same product, and extraneous contamination from microorganisms and personnel. Evaluation of the flow diagrams can assist with strategies for development of engineering controls, personnel procedures, and monitoring systems to enable compliance with Good Manufacturing Practices (GMPs). Analysis of potential risks may also provide insights about what information should be recorded in batch documentation to facilitate consistent manufacture and also to facilitate failure investigations. Together, physical facilities, procedures, personnel, training, and quality systems make up the GMP environment in which a vaccine will be produced.

Manufacturing Process

The manufacturing process includes process inputs such as raw materials and processing aids and unit operations comprising both the initial and downstream processing steps. A process flow map for the manufacturing process is useful and assists in validation of the manufacturing process. This map shows all unit operations, the inputs to each operation, and the outputs to subsequent manufacturing steps. Analytical testing done at relevant steps and the specifications required to proceed to the next stage of processing may be added to the map. A process map also supports a processing space to facilitate a rugged process, i.e., one based on suitable characterization studies to establish boundaries within which manufacturing can occur to promote unchanged safety and efficacy outcomes.

The process flow map should include all steps from making the seed/cell bank (described below) to formulation and filling of the final product. The validation strategy should include the steps that require validation, along with identification of the process space, associated critical process parameters (CPP), and critical quality attributes (CQA). The critical process parameters are those that directly affect core quality attributes needed to successfully manufacture a batch of product. Some manufacturers identify other processing parameters that are important for processing but do not affect critical quality attributes. These important but noncritical factors help to identify the process development space, can contribute to the development of a rugged process, or can be useful when the company assesses processing deviations. The concepts of quality by design and exploration of the process space are relatively new to the biologics/vaccine industry but are becoming considerations for the overall development-planning process.

Manufacturing Surveillance

Manufacturing surveillance is the continual observation of how the process and the resulting product are performing. This section is not exhaustive; rather, the points raised here outline the types of considerations recommended for a manufacturer during development of a vaccine. Manufacturing surveillance includes the following:

- Periodic review of the performance of the manufacturing process;
- Analytical assays;
- Stability programs;
- Product complaints;
- Adverse event reports;
- Product failure investigations;
- Atypical or deviation events.

Taken as a whole, these activities allow a manufacturer to assess the state of the process and product and to evaluate which, if any, operations need to be modified. These same systems also provide a surveillance matrix to evaluate changes. In any of these programs it is also valuable to develop additional characterization assays that are not used for process intermediate or product release purposes but may be used for further evaluation when additional information is needed or desired. These additional assays for characterization are often based on different underlying analytical procedures to provide different ways to evaluate materials.

Routine surveillance processes are increasingly implemented to attempt to detect changes in processes before any critical quality attributes are adversely affected. Not all vaccine processes can be characterized to the same extent or level (e.g., a live virus vaccine vs. a recombinant protein vaccine), and statistical tools are often used to determine alert or action levels in surveillance programs. Exceeding these levels requires the manufacturer to evaluate the situation but does not necessarily signal product failure.

GMP manufacturing entails facility design, process development, quality systems, and manufacturing surveillance. Together these systems help the manufacturer to control the production of a vaccine. As noted, many types of vaccine are marketed, and each has its unique features and therefore requires different plans for each of the steps mentioned in this section.
SEED LOT SYSTEMS

Seed lots are the stocks of specific strains of bacteria, viruses, or biotechnology-engineered cells used to express vaccine antigens. All seed lots should be documented in terms of their isolation, derivation (or construction, in the case of recombinant vector or engineered cells), and passage history. The purpose of a seed-lot system, which typically includes master and working stock seeds, and associated master and working cell banks, is to help ensure the consistency of vaccine manufacturing. The use of master and working seed lots provides a method to limit the replication of the seed and to minimize the possibility of genetic variation.

A master seed lot is a physically homogeneous preparation derived from an original seed processed at one time and passaged for a limited number of times. The master seed lot is characterized for its biological, biochemical and genetic characteristics, and to ensure its purity, its freedom from adventitious agents, and its clinical ability to produce an effective vaccine.

Cultures from the working seed lot should have the same characteristics as the master seed lot from which they are derived. For influenza vaccines, which may be reformulated with new virus antigens each year, certified seed lots can be obtained from national regulatory agencies.

A working seed lot is derived from the master seed within a limited number of passages. The working seed is tested to ensure its purity, freedom from adventitious agents, and biochemical properties. The working seed is used for production of vaccine without intervening passages.

Bacterial Vaccine Seed Lot System

In the bacterial seed lot system, a master seed is subcultured to produce a working seed one passage beyond the master seed. An aliquot of the working seed is then expanded to produce a vaccine lot. The strain(s) used for the master seed lots are identified by historical records that include information about their origin. Information about the bacterial seed lot system should include source, passage history, and raw materials to which it was exposed, with specific emphasis on raw materials of ruminant origin. Seeds should be stored at an appropriate temperature in more than one location within a facility or at a distant site in order to decrease catastrophic risk.

Identity tests may include inoculation onto suitable biochemical media, Gram stains, genotype, and serological identification with suitable specific antisera. Special tests may be added, for example, to show culture viability but also lack of virulence.

Purity of the bacterial strains used for seed lots is verified by methods of suitable sensitivity to ensure that no adventitious agents are present. These purity tests often are performed in the presence of the seed under conditions where growth is inhibited by the presence or the absence of specific nutrients. Streaking can also be used to show that the cultured seed is a pure culture.

Viral Vaccine Seed Lot System

The derivation and passage history of viral seeds should be recorded in detail. Any manipulation of the viral phenotype (e.g., cold adaptation, development of temperature sensitivity, or attenuation of virulence) or intentional genetic manipulations (e.g., reassortment or recombination) should be documented.

These viral seeds are commonly differentiated into a master viral seed and working viral seeds or working viral stock. Viral seeds should be stored at cryogenic temperatures to promote stability and in more than one location within a facility or at a distant site to decrease catastrophic risk. Manufacturers should assess the following characteristics of the viral seed stock:

- Growth characteristics on the intended production cell substrate,
- Tissue tropism;
- Genetic markers;
- Identity (for recombinant vectors);
- Viability during storage,
- Genetic stability through production;
- Attenuation properties;
- Purity;
- Absence of adventitious agents. If attenuation or derivation is achieved by passage through different species, the viral seed should be assessed for absence of adventitious agents common to those species.

The master viral seed should be extensively characterized to demonstrate the stability of genotype and phenotype for a number of passages beyond the level used in production. Generally, during assessment of genetic stability, a master seed undergoes a minimum of five passes beyond the passage that will produce the final vaccine.

Tests should be performed for identity (e.g., sequencing the entire virus or a portion of it), adventitious agents, viral phenotype, genetic stability, and, if applicable, agents that might be present in the seed as a result of its passage history. Viral phenotype can be assessed further for tissue tropism, attenuation properties, and temperature sensitivity. Not all of these tests may be necessary for every viral seed strain.

In some cases the viral seeds may have a broad host range and therefore may require neutralization of the vaccine virus before they are tested for adventitious agent(s). If possible, testing for adventitious agents should be done without neutralization in order to avoid an antiserum that may inadvertently neutralize an adventitious agent present in the seed. Sometimes it is not possible to effectively neutralize a viral seed, and in such cases alternative strategies can be used. For example, the test can be performed in a cell substrate that does not permit replication by the vaccine virus. However, such a substitution of the substrate cell may compromise the test's sensitivity for detection of other adventitious agents. Therefore, the tests may be supplemented with use of polymerase chain reaction (PCR) assays.

Assessment of neurovirulence may be appropriate if the virus is known to be neurotropic. Manufacturers should consult with regulators about appropriate animal models, methods, and scoring systems for this assessment before they initiate such studies.
For viruses that are neurovirulent or may revert to neurovirulence (e.g., polioviruses), it may be necessary to assess neurovirulence beyond the master seed.

If the master viral seed is well characterized, the working viral seed may not require extensive characterization. For example, it may not be necessary to repeat testing for all the relevant viruses from the derivation history.

**Systems for Biotechnology-Engineered Vaccines**

For a vaccine produced via a biotechnology-engineered cell-expression system, a master seed lot or a master cell bank will be established during product development. The seed lot or cell banks should be homogenous, which is often accomplished by limiting dilutions. The seed lot or cell bank system should be characterized in a manner analogous to that used for the cell substrate discussed in the next section, and additional tests can be used to demonstrate the genetic stability of the expression system.

**FERMENTATION AND CELL CULTURE MEDIA**

A medium is the material in which an organism is grown and amplified in quantity to produce mass material for vaccine production. Its composition is diverse and depends on the cell types that the medium supports, ranging from well-defined chemical media to chemically undefined media that contain natural components such as sera from animal origin (see Bovine Serum). Culture media should be suitable for their intended purpose and should be free from adventitious agents and known undesirable components such as toxins, allergens, and similar compounds. If undefined ingredients are necessary, the amount should be kept below levels that are demonstrated to be safe for the final product.

**Fermentation Media for Bacterial Growth**

The nutrients consist of materials like proteins, sugars, inorganic trace elements, amino acids, and vitamins needed for bacterial growth. The protein component may be as simple as free casein (milk protein), or it can be as complex as extracts from bacterial, plant, or animal sources. Any fermentation nutrients of animal origin are sourced carefully and tested for adventitious agents. The composition of a medium is often customized to optimize product quality attributes. Medium components that are known to cause allergic reactions should be avoided.

**Media for Cell Culture for Viral Vaccines**

The types and composition of media used for isolation and all subsequent culture of components of viral vaccines need to be recorded in detail. Chemically defined media without materials of animal origin are preferred. The medium should be tested for sterility and suitability for the cells used in product production. If materials of animal origin are used, they are assessed for freedom from adventitious agents. If human albumin is used in a U.S.-licensed vaccine, it must be licensed by FDA. The final product should be within specified limits of residual medium components such as serum, antibiotics, selection agents or reagents added for growth enhancement.

**Media for Biotechnology-Engineered Cells**

The requirement for media used for the fermentation and propagation of biotechnology-engineered cells is the same as that noted above for bacterial fermentation and cell culture growth.

**PROPAGATION AND HARVEST**

The propagation and harvest phases follow the manufacturing process from the initiation of cell growth in the working cell bank to the separation of the crude drug substance. In addition, in these manufacturing process steps, raw materials, media, and solutions should be qualified for their intended use. Batch numbers should be clearly assigned as needed, and the relationship between component harvests and batches of individual drug substances should be recorded clearly.

**Propagation and Harvest for Bacterial Vaccines**

Propagation of bacteria for bacterial vaccines is performed under specified conditions for the inoculum preparation and the fermentation phases. In-process monitoring and testing should be conducted for quality assurance. All controls and testing performed after production (e.g., purity, viability, antigen yield, and phenotypic identity) should be documented. The first step of drug-substance recovery is harvesting from the bioreactor. A variety of equipment is available, and the process equipment used depends on the nature of the process. Procedures should be established to ensure containment and prevention of contamination during harvesting and to monitor bioburden (including acceptance criteria) or sterility. The storage conditions and the stability time limit for the harvest material should be described. For most bacterial vaccines, an inactivation step is necessary. Personnel involved in bacterial inactivation should consider the following: how cell culture purity is verified after inactivation, whether culture purity should be defined before inactivation, choice of the inactivation agent, and validation of the procedure(s).
Propagation and Harvest for Viral Vaccines

The manufacturing of viral vaccines using eukaryotic cell culture includes a two-phase production process. The first is the expansion of the cell cultures used as a substrate for viral replication. The second phase includes the initial virus infection and subsequent replication and virus production.

**CELL SUBSTRATE GROWTH PHASE**

The cell substrate expansion process for viral production is the phase designed to prepare the cells in a physiological state appropriate to sustain virus growth. Cell substrates often require complex animal-derived supplements such as serum. The source and testing requirements of bovine serum are subject to regulatory requirements (see Bovine Serum á 1024).

**VIRUS PRODUCTION PHASE**

Relatively few cell types have been used as substrates in U.S.-licensed viral vaccines, but these include primary cells (e.g., certain cells derived from monkey, chick, or mouse tissue), diploid cell lines (e.g., WI-38, MRC-5, or FRhL-2), and continuous cell lines (e.g., Vero). Vaccine manufacturers have optimized nutrient requirements, growth factors, and serum concentration to support robust growth and strong virus productivity for these cell lines.

**PURIFICATION**

The objective of the purification steps is to remove as much as possible of the impurities in the initial harvest and to maximize the purity of the final vaccine product. Process residuals may consist of materials from the culture medium and/or cellular components. Purification procedures should be optimized and validated. When applicable, viral clearance steps (viral removal or inactivation) should be included and validated using relevant model viruses. Special considerations are observed depending on the types of vaccines and production system used, as discussed below.

**Bacterial Fermentation**

Bacterial fermentations are typically highly productive and yield large amounts of biomass. For bacterial subunit products or recombinant components expressed by bacteria, fermentation can produce very high concentrations of the desired active ingredient. Manufacturers should initiate culture purity testing before further processing.

**LIVE BACTERIAL VACCINES**

Live bacterial vaccines such as Bacillus Calmette-Guérin (BCG) and Salmonella typhi Ty21a are relatively fragile as pharmaceutical products and therefore tolerate only fairly gentle purification approaches. If osmotic and shear forces are constrained, then the integrity of the bacteria usually can be maintained.

**INACTIVATED BACTERIAL VACCINES**

At present no inactivated whole-cell bacterial vaccines are licensed for use in the U.S.

**PURIFIED BACTERIAL ANTIGENS**

Purification of bacterial components (e.g., proteins, toxins, and polysaccharides) generally requires cell disruption. More selective purification methods can be used to remove culture media and bacterial impurities and to achieve high purity of the target bacterial component.

**Biotechnology-Engineered Cells**

Of special concern in the purification of recombinant-derived vaccine components is the issue of residual host cell components that could produce an adverse immunogenic response in patients. This response could be exacerbated by the presence of vaccine adjuvants.

**RECOMBINANT VIRUS-LIKE PARTICLES (VLP)**

Formation of VLPs can coincidentally result in incorporation of host cell components (e.g., DNA) into the quaternary structure of the molecular assembly, resulting in a class of impurities that has a tight association with the active pharmaceutical ingredient. As a result, modern approaches to VLP production in some cases include a disassembly step that dissociates impurities from the viral proteins. This procedure is followed by a reassembly step that reforms the VLPs in the absence of the host components. Liquid-phase extractions and chromatographic procedures can be used to provide high-purity components for use in vaccine products with no substantial risk of carrying over significant residual host components.
Viral Vaccines Derived from Cell Culture

VIRAL VACCINES DERIVED FROM CONTINUOUS CELL LINES

If a continuous cell line (e.g., Vero) is used for vaccine production, a validated filtration step is necessary to separate virus from intact cells. The quantity and size of any residual host cell DNA also should be determined (see general information chapter Nucleic Acid-Based Techniques—Approaches for Detecting Trace Nucleic Acids (Residual DNA Testing) (1130)). Currently, 10 ng of host cell DNA is permitted per dose of a parenterally administered vaccine, and regulatory agencies continue to consider on a case-by-case basis the level of risk posed by host cell DNA for vaccines that are administered by other routes (e.g., nasal or oral). Multiple purification methods to reduce the size and amount of residual host-cell DNA present in the vaccine are desirable and include steps such as treatment with DNase, diafiltration, ultrafiltration, and column chromatography.

VIRAL VACCINES DERIVED FROM HUMAN DIPLOID CELL CULTURE

FDA has licensed several vaccines made using human diploid cells. The two most commonly used diploid cell lines are MRC-5 and WI-38, both of which are derived from human embryonic cells and have the normal diploid number of human chromosomes. They are widely used to manufacture vaccines because they have been shown to have no tumorigenic or oncogenic potential and have been shown to be susceptible to a wide range of human viruses. However, unlike continuous cell lines that can be passaged indefinitely, human diploid cell lines are capable of attaining only a certain number of population doublings, after which they experience a rapid decline in their ability to proliferate. This issue is managed by freezing multiple aliquots of master and working cell banks.

VIRAL VACCINES DERIVED FROM PRIMARY CELL CULTURE

Like diploid cells, primary cells normally are not tumorigenic or oncogenic. However, when primary cells are used to manufacture live vaccines, the donor animals from which the primary cells are obtained are extensively tested for a variety of pathogens before being used. For example, chicken flocks used to prepare chicken embryo kidney cells undergo extensive serological testing for adventitious agents before the flock can be used to prepare the cells. Some of these tests are described in the Code of Federal Regulations (CFR, see the sections listed in Appendix 2) and the USP general information chapter Virology Test Methods (1237).

Viral Vaccines Derived from Chicken Eggs

The embryonated chicken egg is a highly productive growth substrate for certain viruses, such as those used to make vaccines for yellow fever and several influenza vaccines. In the case of influenza vaccines, vaccine virus is harvested from egg allantoic fluid. In the case of yellow fever vaccine, the vaccine virus is harvested from embryo tissues. Therefore, residual egg or embryo components are special considerations in vaccine purification.

Egg-based vaccine production, like all biomass expansions, requires care and quality control of the virus seed lots and egg substrates to avoid contamination with other organisms.

LIVE ATTENUATED VIRUS VACCINES

Viruses for live vaccines (e.g., yellow fever or live influenza) are produced using Specific Pathogen-Free (SPF) eggs. These eggs are produced by chicken flocks that are regularly screened for avian pathogens (e.g., avian leukemia virus) and are maintained using appropriate animal husbandry practices. To preserve the infectivity and antigenic integrity of the vaccine viruses while removing egg-derived components, relatively simple, mild methods (e.g., zonal sucrose gradient centrifugation and diafiltration) are used for vaccine virus concentration, purification, and buffer exchange.

INACTIVATED WHOLE VIRUS VACCINES

Viruses for inactivated vaccines can be produced using non-SPF eggs because of required chemical inactivation steps in the manufacturing process. Because the vaccine virus needs to be retained intact while removing egg-derived components and inactivating chemicals, relatively mild purification and concentration methods (e.g., zonal sucrose gradient centrifugation) are used. If chemical agents are used in the process, they should be minimized in the final product to below prespecified levels.

SPLIT VIRUS AND PURIFIED SUBUNIT VACCINES

Viruses for split virus and purified subunit influenza vaccines are produced in non-SPF embryonated eggs. Inactivation and purification of vaccine viruses are achieved by chemical treatment (e.g., formaldehyde or β-propiolactone) and zonal sucrose gradient centrifugation, respectively. Split virus vaccines are prepared by disruption of vaccine virus particles using a detergent (e.g., sodium deoxycholate) that preserves antigenic integrity.

INTERMEDIATES

Intermediates are defined here as the unformulated active (immunogenic) drug substances that are processed before final formulation and can be stored for long periods of time before further processing. These intermediates can be stored and should be included in a formal stability program. Examples of intermediates include bulk polysaccharides, purified recombinant proteins (concentrates), and conjugates.
Production of Intermediates

Intermediates are manufactured from starting materials by one or a combination of different processes (e.g., fermentation, cultivation, isolation, or synthesis). Subsequent steps of the procedure involve preparation, characterization, and purification, eventually resulting in the drug substance. Quality systems documents are adopted for production and all applicable information should be recorded in a controlled document (i.e., a batch record). When applicable, stability studies and release tests should be performed before proceeding to the next steps (see below).

Tests for Intermediates

The quality attributes of the intermediate are commonly tested in conjunction with further processing. Characterization beyond release testing should be considered. Characterization methods can use appropriately qualified procedures. Some tests are routinely performed before the intermediates are converted to the final bulk, depending on individual vaccines.

If intermediates need to be stored and/or subsequently shipped to a different location for further processing, the stability of these materials should be demonstrated. Stability tests can be a combination of both physicochemical analysis and biological assays.

FINAL BULK

Final bulk is the bulk drug product that contains the drug substance(s), excipients, and other ingredients at desired concentrations and is ready for filling into individual containers.

Production of Final Bulk

Appropriately controlled amounts of all ingredients are blended to uniformity to produce the final bulk. The processing may include one or more steps such as buffer exchange and addition of diluents, bulking agent, stabilizing excipients, adjuvants, and preservatives. Final bulk may be prepared aseptically or processing may include a sterilization step.

Tests for Final Bulk

The quality attributes of the final bulk should be tested. Appropriate testing should be performed with respect to identity, purity, potency, sterility (see Sterility Tests (71)), and antimicrobial effectiveness (see Antimicrobial Effectiveness Testing (51)). Tests demonstrating safety, if applicable, are performed. The list includes, for example, tests for the absence of adventitious agents, mycoplasma, and other microorganisms.

Testing is required for specific process-related and product-related impurities, depending on the vaccines being manufactured. In addition, tests are required for the bulking agent, stabilizing excipients, adjuvants, and/or preservatives, if used. All the testing should be done according to respective standard operating procedures (SOPs), and all tests should have specifications (or provisional specifications, where applicable).

Stability Test for Final Bulk

If final bulks are stored and/or subsequently shipped to a different location for further processing, the stability of these materials should be demonstrated. Stability tests can be a combination of both physicochemical analysis and biological assays. Implementation of a stability program is required for formal stability studies, and the studies should be executed according to a protocol that contains detailed information about types of tests, including specifications, testing intervals, and data and analysis.

FINAL CONTAINER

A final container of vaccine contains the active ingredient(s) (i.e., antigen(s)) as well as additional components, such as stabilizers, adjuvants, or antimicrobial preservatives. They also may include residual materials from the manufacturing process.

Excipients and Other Additives

In addition to specific antigens, vaccines often include excipients and other additives that are intentionally added to the vaccine by the manufacturer for a specific purpose. These include adjuvants, antimicrobial preservatives, and stabilizers. Vaccines also contain manufacturing residuals, which are trace amounts of various components used during manufacturing. Thus, the combinations of these components comprise and define the complete vaccine product. Manufacturers must adhere to regulations governing permissible limits of such components, as indicated in the product’s license.

ADJUVANTS

Adjuvants are agents incorporated into vaccine formulations to enhance and increase the immune responses generated by the vaccine antigens. Specifically, they can increase the amount of antibody produced, direct the immune response (Th1 or Th2), increase the duration of antibody presence (persistence), or produce a combination of these effects.
Aluminum compounds have long been the most widely used adjuvants worldwide. Two methods traditionally have been used for combining aluminum adjuvant to antigen to form aluminum-adsorbed vaccines. The first involves the addition of the antigen solution to preformed aluminum precipitate. The second involves the addition of an antigen to aluminum in solution and the addition of a compound that will coprecipitate the aluminum salt and the antigen in situ. Solutions of aluminum potassium sulfate, known as alum or aluminum chloride, have been used together with phosphate salts as precipitating agents. A number of aluminum adjuvant formulations are used in vaccines. Tests for aluminum are based on metal detection tests described in the general test chapter Aluminum (206). Regulations limit the amount of aluminum permitted in a dose of vaccine. The Code of Federal Regulations [21 CFR 610.15(a)], Ingredients, preservatives, diluents, adjuvants] states that “the amount of aluminum in the recommended individual dose of a biological product shall not exceed:

1. 0.85 milligrams if determined by assay;
2. 1.14 milligrams if determined by calculation on the basis of the amount of aluminum compound added; or
3. 1.25 milligrams determined by assay provided that the data demonstrating that the amount of aluminum used is safe and necessary to produce the intended effect and are submitted to and approved by the Director, CBER [Center for Biologies Evaluation and Research at FDA].”

The third criterion above aligns U.S. regulations with World Health Organization guidance for aluminum content in a single human dose of a vaccine product. Note that adjuvants are not licensed by themselves; they do not constitute a product. Rather, a vaccine consisting of specific antigen(s) and an adjuvant are licensed together as a drug product.

ANTIMICROBIAL PRESERVATIVES

In the case of multiple-dose containers, antimicrobial preservatives are added to inhibit the growth of microorganisms that may be introduced from repeated puncture of multidose vials. With certain exceptions, a preservative is required to be present in vaccines marketed in multidose containers [21 CFR 610.15(a)]. Exceptions include yellow-fever vaccine; measles, mumps, and rubella (MMR); and dried vaccines when the accompanying diluent contains a preservative.

The microbial preservatives currently used in vaccines are thimerosal, 2-phenoxyethanol, benzethonium chloride, and phenol. These agents must pass the appropriate antimicrobial effectiveness test, as described in Antimicrobial Effectiveness Testing (51). Antimicrobial test challenges should be conducted as part of the normal formal stability program, including at expiration date. Various tests for preservatives can be found in Antimicrobial Agents—Content (341).

STABILIZERS

The primary purpose of stabilizers is to protect certain vaccines from adverse conditions such as heat or to serve as a cryopreservative during the lyophilization process, usually the freezing step. The particular materials chosen for this purpose include sugars (e.g., sucrose or lactose), amino acids (e.g., glycine or glutamic acid [monosodium salt]), glycerol, and proteins (e.g., human serum albumin [HSA] or gelatin). Materials should be customized to a specific vaccine formulation and selected with patient safety in mind. When a protein is chosen as a stabilizer, two main safety concerns arise. One stems from the source of the protein: animal or human origin raises the possibility of the presence of an adventitious agent. The second concern is the possibility of an allergic reaction in persons sensitized to that protein. This should be evaluated as part of the clinical program during vaccine development. At present two proteins are used as stabilizers for vaccines: HSA and gelatin. FDA requires that any serum-derived albumin used in manufacturing be U.S.-licensed HSA. FDA guidance further recommends that a statement indicating the source and related risks appear in the “Warnings” section of the labeling for HSA-containing products.

Gelatin or processed gelatin also is used as a vaccine stabilizer. The gelatin source may be either bovine or porcine. Although the conditions of manufacturing gelatin are harsh (i.e., the product is subjected to extremes of heat and pH), there remains a concern with bovine sources about the presence of the transmissible spongiform encephalopathy (TSE) agent, because this agent is known to resist such conditions. Therefore, if gelatin added to a vaccine or used in manufacturing is from a bovine source, the material should have the appropriate documentation certifying that it comes from a country or region that is in compliance with TSE guidance for industry.

MANUFACTURING RESIDUALS

Vaccines may contain residual amounts of any of the materials used in the manufacturing process. These materials are termed manufacturing residuals. As a general principle, it is not possible to remove a particular substance completely, nor is it possible to conclusively demonstrate that a particular substance has been completely removed. Therefore the goal is to reduce these substances to an undetectable level, using a sensitive and validated analytical methodology. Some products are tested for pyrogenic substances as a manufacturing residual (see Pyrogen Test (151)); and, if the product is freeze-dried, it should be tested for residual moisture (see Loss on Drying (731)). Residual levels of manufacturing materials, including, if applicable, inactivating agents, should be justified. The release specifications of these components are required as part of the approved license.

CELL-DERIVED RESIDUALS

Live attenuated bacterial vaccines are not usually subject to a high degree of postexpansion purification. But killed bacterial component vaccines typically undergo significant purification to reduce cell-derived residuals. Common cellular components to be reduced are proteins, nucleic acids, and polysaccharides. Assays for these components are routinely conducted, if appropriate, to ensure purity. A common residual in bacterial vaccines made from Gram-negative bacteria is lipopolysaccharide (LPS), commonly known as endotoxin. Endotoxin testing is performed during the manufacturing process for any Gram-negative bacterial vaccine. In the case of Gram-positive bacterial vaccines, the endotoxin testing should be conducted to ensure that no
CONTAMINANTS FROM GRAM-NEGATIVE BACTERIAL GROWTH ARE PRESENT. ALSO, THERE MUST BE A RELEASE SPECIFICATION FOR THIS RESIDUAL. TWO TESTS ARE CURRENTLY USED TO DETECT LPS IN BIOLOGICAL PRODUCTS, THE \textit{Limulus} amebocyte lysate (LAL) test (see \textit{Bacterial Endotoxins Test} (85)) and the rabbit pyrogen test (see \textit{Pyrogen Test} (151)). The \textit{Limulus} lysate that is used is typical for bacterial endotoxin in FDA-regulated products is itself a U.S.-licensed product. The rabbit pyrogenicity test requires the use of animals and is more difficult to perform; therefore, it is not employed to the extent that the LAL test is used.

Viral vaccine manufacturing requires cell substrates to produce the viruses, which are then taken through purification processes. Generally, killed viral vaccines are more highly purified than are live attenuated ones. Depending on the method used to manufacture the vaccine, manufacturers work with FDA to develop prudent specifications for the final vaccine. Animal-derived host cells have been used extensively in vaccine manufacturing, particularly viral vaccines. For example, influenza and yellow fever vaccines are produced, respectively, in egg allantoic fluid and chicken embryos. Mumps, measles, and some rabies vaccines are produced in chick embryoid cells. The labels of these products must state that residual chicken proteins may be present in the final vaccine, and the label may indicate how much is present. Further, the label also urges practitioner caution when vaccinating a person with known hypersensitivity to eggs.

Two U.S.-licensed hepatitis B vaccines are based on recombinant DNA-derived proteins expressed in yeast cultures. In both cases, the labels notify health care professionals that yeast protein may be present in the vaccine and recommend that suitable precautions should be exercised. In the case of live viral vaccines, considerations may be given to the reduction of cellular residual materials (e.g., host DNA, proteins).

\section*{MATERIALS OF ANIMAL ORIGIN}

Some raw materials and reagents, such as gelatin, calf serum (see \textit{Bovine Serum} (1024)), or trypsin for vaccine manufacturing raise concerns regarding the potential presence of adventitious agents. Raw materials should be sourced from countries acceptable to FDA. Additionally, manufacturers should test these materials when possible to minimize the risks of contamination with adventitious agents. Reduction of serum components (e.g., BSA) should be considered in processing.

\section*{ANTIBIOTIC RESIDUALS}

Some antibiotics (but not penicillin) can be used in minimal amounts in the manufacturing process for viral vaccines, according to 21 CFR 610.15(c). Those that have been used include gentamicin, streptomycin, neomycin, and polymyxin B. There is no requirement for tests of residual levels of these antibiotics in the final vaccine. However, according to 21 CFR 610.61(m), the calculated amount expected to remain as a residual in the final vaccine, based on the amount added and the dilution factor in the manufacturing process, must be stated on the product label.

\section*{INACTIVATING CHEMICAL AGENTS}

Several chemical agents have been used to inactivate bacteria and viruses or to detoxify toxins in vaccine production processes. Formaldehyde and \textit{β}-propiolactone are the most commonly used inactivating agents. Other less often used inactivating agents include glutaraldehyde and hydrogen peroxide. As a manufacturing residual, the inactivating agent should be removed from the final product as thoroughly as possible. The upper limit for formaldehyde is generally 0.02%, equivalent to 0.1 mg per 0.5-mL vaccine dose. The limit for \textit{β}-propiolactone should be below the limit of detection.

\section*{EVALUATING THE STABILITY OF VACCINES}

The stability of vaccine products depends on the nature of a vaccine antigen, the product formulation, and the control of vaccine storage prior to use. Vaccine products are evaluated with programs that include real-time long-term storage under prescribed conditions. The use of extreme temperatures to potentially accelerate degradation may help manufacturers understand the stability of the product.

Vaccine products, like all pharmaceutical products, should be evaluated to define suitable conditions for storage (21 CFR 610.50 and 610.53). General principles of stability testing for biological products are described in \textit{Quality of Biotechnological Products: Stability Testing of Biotechnological/Biological Products} (1049). Typically these concerns are focused on the final vaccine product, but evaluations also are needed for bulk intermediates to justify the conditions under which they are kept. In both cases manufacturers define in advance the conditions to which the product will be exposed (e.g., temperature, light, and humidity) and the time range during which the product will be exposed to those conditions. Stability studies should evaluate all storage conditions to which the product or intermediate is likely to be exposed during production, handling, shipping, and storage so that appropriate time limits can be placed on the exposure to those conditions.

The primary criteria for defining the storage conditions for these intermediates and the final products are generally focused on acceptable maintenance of potency; but, as discussed below, there often are other attributes that need to be considered. Evaluation of the stability of vaccine products has three general purposes. First, the products are shown to maintain an acceptable analytical profile throughout manufacture and use to preserve safety and effectiveness. Second, stability studies across several product batches provide an effective way to characterize the inherent properties of the product. This in turn leads to the third use, demonstrating manufacturing consistency in the product.

\section*{Stability Protocols}

The overall experimental plan for evaluating the stability profile of a given set of product or intermediate batches typically includes specific definition of the conditions under which the samples will be stored and why these conditions are relevant. The length of time the samples will be stored at each condition, when samples will be tested during this time course, and the...
analytical measurements at each time point. Additionally, these stability protocols include itemization of the analytical procedures to be used. For stability studies that occur early in product development, the studies may be conducted to confirm the suitability of the product formulation and/or storage conditions. Later in development, stability studies are typically conducted to provide data supporting product dating period or intermediate hold time, to provide more elaborate product characterization, and to evaluate manufacturing consistency. These latter studies define product end–expiry specifications that allow definitions of acceptable and unacceptable product. Unacceptable product is defined as product that is no longer acceptable for use in clinical studies or for commercial use (e.g., because of degradation or loss of potency). Stability studies should be conducted over a duration sufficient to determine the point of loss of acceptable potency or other relevant parameters.

### Analytical Measurements

Manufacturers should consider the rigor of the analytical method(s) used to evaluate the stability of complex products and improve their understanding of the parameters that are critical to immunogenicity (including stability-indicator parameters). Selection of the stability-indicator parameters varies with each vaccine’s unique characteristics.

The primary parameter that reflects stability for most vaccines is the potency assay (see Potency Tests in Lot Release Testing, below). This assay can take many forms, depending on individual vaccines (e.g., an infectivity assay for a live virus vaccine or a measure of the proportion of conjugated polysaccharide for a polysaccharide–protein conjugate vaccine). The potency assay is generally the key analytical result predicting whether a vaccine remains suitable for use and whether it will produce the expected clinical response. Other analytical measurements can provide important supplemental data, particularly those that have a clear link to the potency of the product. Examples include degradation profile, dissociation of a carrier protein from conjugated vaccines, and dissociation of an adjuvant from an antigen complex. Additionally, other common assays typically are performed as part of the stability study and may address physical or chemical changes in the product that may or may not affect its potency (e.g., general safety, degree of aggregation, pH, moisture, container, preservative, and enclosure).

### Formal Evaluation of Stability Data and Product Expiry Dating

Vaccines must remain within potency specifications at the expiration date, provided that the product was stored under the normal conditions specified. Manufacturers should conduct stability studies to determine those storage conditions and that dating period to demonstrate that the product remains within the potency specifications. Manufacturers should conduct stability studies on a continuing basis. If a major manufacturing process changes, additional stability studies should be conducted to verify that there is no adverse impact on the stability profile. Under certain conditions such as process changes, accelerated stability studies could be conducted. An accelerated study involving temperatures both higher and lower than routine can evaluate the impact of temperature excursions on products. A similar evaluation should be done for product intermediates to establish how long a given intermediate can be held under defined conditions before it is processed further or discarded.

### NOMENCLATURE

There are no uniform systems for naming new vaccines. 21 CFR 299 describes the cooperation of the FDA and the U.S. Adopted Names Council (USAN) in naming drugs, including vaccines. USAN is a private organization sponsored by the American Medical Association, USP, and the American Pharmacists Association. Section 262 in Title 42 of the Public Health Service Act requires that each package of the biological product be plainly marked with the proper name (name designated in the license 21 CFR 600.3) of the biological product contained in the package.

### LABELING

Vaccine product labeling is regulated in compliance with 21 CFR 201 and 610. Requirements are set for container labeling and package labeling.

#### Container Label

Provisions are made for the following labels:
- Full label;
- Partial label; and
- No label on the container itself when the containers cannot support a label that includes all required information and should be placed in a package that does include all required information.

The label should be affixed to the container in a manner that allows visual inspection of the contents for the full length or circumference of the container. If no package exists, the container bears all of the information required for the package label. The full container label normally contains the following:
- Proper name of the product;
- Name, address, and license number of the manufacturer;
- Lot number or other lot identification;
- Expiration date;
- Recommended individual dose, for multiple-dose containers;
- The phrase Rx only for prescription biologicals; and
- Any applicable cautionary statements.
Package Label

In addition to the information required on the container label, the package label should describe the following:

- Any preservative used and its concentration, or the words *no preservative* if no preservative is used and its absence is a safety factor;
- Number of containers, if more than one; or
- Amount of product in the container, expressed as number of doses, volume, units of potency, weight, and equivalent volume (for dried product to be reconstituted); or
- A combination of the above to provide an accurate description of the contents, as applicable;
- Recommended storage temperature;
- The words *shake well, do not freeze*, or the equivalent, as well as other instructions when indicated by the character of the product;
- Recommended individual dose, for multiple-dose containers;
- Recommended route of administration, or reference to such directions in an enclosed circular;
- Presence of known sensitizing substances;
- Type of antibiotics added during manufacture and the amount calculated to remain in the final product;
- Inactive ingredients, when they constitute a safety factor or are referenced to an enclosed circular;
- Adjuvant, if present;
- Source of the product, when this may be a factor in safe administration;
- Identity of each microorganism used in manufacture and, if applicable, the production medium and the method of inactivation or reference to an enclosed circular;
- Minimum potency in terms of official standard of potency, or the words *no U.S. standard of potency*.

Prescribing Information

Detailed information about a vaccine appears in its prescribing information, commonly called the package insert. Increasingly, vaccines are distributed with patient package inserts written in lay language. Prescribing information (21 CFR 201.56 and 201.57) includes the following:

- Highlights of prescribing information
- Product names, other required information
- Boxed warning
- Recent major changes
  1. Indications and usage
  2. Dosage and administration
  3. Dosage forms and strengths
  4. Contraindications
  5. Warnings and precautions
  6. Adverse reactions
  7. Drug interactions
  8. Use in specific populations (e.g., pregnancy, nursing mothers, pediatric, geriatric)
  9. Drug abuse and dependence
  10. Overdosage
  11. Description
  12. Clinical pharmacology
  13. Nonclinical toxicology
  14. Clinical studies
  15. References
  16. How supplied/storage and handling
  17. Patient counseling information

LOT RELEASE TESTING

General Principles

Manufacturers perform all appropriate tests for the licensed specifications for the product, according to 21 CFR 610.1 and 610.2. Samples of each licensed lot and protocols containing the manufacturers’ test results are submitted to FDA. After FDA evaluates the protocol to ensure that the product specifications are met, and after satisfactory confirmatory testing, FDA approves the release of the lot if all tests meet the standards of safety, purity, and potency established for the particular vaccine product. After approval is granted, the manufacturer distributes and markets the product.

Guidelines are available regarding alternatives to lot release and a surveillance system. All of these variations are subject to the regulations in 21 CFR 610.2 that allow FDA to require that samples of any lot of licensed product (e.g., vaccine), together with the protocols showing results of applicable tests, be sent to FDA.
Common Tests

The tests common to all lots of all products include tests for potency, general safety, sterility, purity, identity, and constituent materials. The manufacturer completes these tests for conformity with standards applicable to each product. The results of all tests are considered, except when a test has been invalidated as a result of causes unrelated to the product (21 CFR 610.1).

POTENCY TESTS (VACCINE-SPECIFIC)

The basic definition and requirements for vaccine potency and potency assays are provided in 21 CFR 600.3 and 610.10. A vaccine potency assay should indicate the therapeutic activity of the drug product as indicated by appropriate laboratory tests or by adequately developed and controlled clinical data. Potency may be expressed in terms of units by reference to a standard. Product potency tests vary with vaccine product types (e.g., viral, bacterial, live attenuated, inactivated, or polysaccharide). As a result, potency assays for vaccines span a variety of approaches to the expression of potency. In vitro potency tests for live virus may include plaque formation assays, endpoint dilution assays (e.g., the tissue culture infective dose [TCID\text{\textsubscript{50}}]), virus neutralization assays, or quantitative polymerase chain reaction [PCR] assays). Quantitative colony formation assays are used for live attenuated bacterial vaccines. Animal challenge tests for immunogenicity assays of potency, such as those for diphtheria and tetanus (U.S. Department of Health, Education, and Welfare, 1953; see Appendix 2), or rabies and anthrax show in vivo response. Antigenicity assays use enzyme-linked immunosorbent assays (ELISA), e.g., with hepatitis A or rate nephelometry and rocket immunoelectrophoresis (e.g., with pneumococcal polysaccharides). The potency tests for bacterial vaccines, such as the meningococcal polysaccharides, pneumococcal polysaccharides, or \textit{Haemophilus b} protein conjugate vaccines use chemical and physical chemical assays. In the case of pure polysaccharide vaccines, the concentration or quantity of the vaccine component (polysaccharide) and its quality (e.g., size) have been shown to be indicative of human immune response. Assay precision and reproducibility vary with the different methodologies that are used in potency assays, ranging from the high accuracy and precision of chemical tests at one end of the spectrum to bioassays at the other end. The general test chapter \textit{Design and Analysis of Biological Assays} (111) provides guidance for bioassays and applies to vaccine potency assays. Other tests should be validated as described in the general information chapter \textit{Validation of Compendial Procedures} (1225).

RELEASE TESTS

Official release of vaccines by the vaccine regulatory authority may be based on either the bulk or the final container. It is highly desirable to perform potency tests on the final container. However, under certain circumstances this may not be practical or even possible; thus, a case-by-case approach would be required. The choice of whether to test the bulk or the final container derives from a number of considerations, such as the quantity of vaccine available for tests at the different manufacturing stages. For certain vaccines, both bulk and final container receive official release. The potency test is generally required for the final container. If it is not feasible to perform the potency test on the final drug product, the test is performed on the bulk material.

GENERAL SAFETY

For biological products that are intended for administration to humans, manufacturers perform a general safety test in order to detect any extraneous toxic contaminants. Procedures and exceptions are specified in 21 CFR 610.11.

STERILITY

A sterility test of each lot of each product is conducted according to procedures described in \textit{Sterility Tests} (71) and 21 CFR 610.12 for both bulk and final container material.

BACTERIAL ENDOTOXINS

Each lot of final containers of a vaccine intended for use by injection is tested for bacterial endotoxins, as indicated in \textit{Bacterial Endotoxins Test} (85).

PURITY

Vaccines need to be free of extraneous material. Approved vaccine license applications indicate extraneous materials that are unavoidable in the manufacturing process for a specific product. The application may indicate test results and allowable limits for such materials, according to procedures described in 21 CFR 610.13.

RESIDUAL MOISTURE

Each lot of dried product is tested for residual moisture [see 21 CFR 610.13 (a), \textit{Loss on Drying} (731), and FDA's \textit{Guideline for the Determination of Residual Moisture in Dried Biological Products} (see Appendix 2)].

PYROGENS

Each lot of final containers of a vaccine intended for use by injection is tested for pyrogenic substances, as indicated in \textit{Pyrogen Test} (151) and 21 CFR 610.13 (b).
IDENTITY

The contents of a final container of each filling of each lot are tested for identity after labeling is completed. Identity is established by physical or chemical characteristics of the vaccine, inspection by macroscopic or microscopic methods, specific cultural tests, or in vivo or in vitro immunological tests. In large part, identity tests are performed to distinguish the subject vaccine from other materials manufactured at the same site (21 CFR 610.14).

CONSTITUENT MATERIALS

Ingredients, preservatives, diluents, adjuvants, extraneous protein, cell culture-produced vaccines, and antibiotics are tested according to 21 CFR 610.15.

Permissible Combinations

Formulations that combine several vaccines must be licensed as combinations (21 CFR 610.17). The potency of each vaccine in the combination is individually tested and must meet the specifications in the context of the final combined product; other appropriate quality tests apply as well. For vaccines that are physically combined in clinical locations just before administration to a patient, prescribing information should describe specific procedures to follow in those settings.

Quality

In general, quality control systems for vaccine manufacture are identical to those routinely employed for production of other pharmaceuticals. These include raw material testing and release, manufacturing, process-control documentation, and aseptic processing. Manufacturers formally assign responsibility to designated staff for maintaining the continued safety, purity, and potency of the product and for ensuring compliance with applicable product and establishment standards, along with compliance with current GMPs. Analysts use reference standards and validated methods to determine active ingredients, residuals, and impurities. Manufacturers determine product safety in a variety of ways that may include the use of experimental animals, procedures to demonstrate product sterility, and tests to ensure product potency. The complexity of the quality control systems for vaccines lies in the variety of methods used to produce and control production. Lot release testing proceeds according to 21 CFR 610.2 and involves evaluating lots for safety, purity, and potency before release. Manufacturers follow FDA and applicable international standards for testing and validation. The basic considerations for validation are included in Validation of Compendial Procedures (1225), in addition to guidance documents issued by FDA and the International Conference on Harmonization (ICH) (see Appendix 2).

Alternative Tests

Modification of test methods or manufacturing processes as licensed may be permitted if the regulatory authority can be assured that the modifications cause no reduction in safety, purity, potency, and effectiveness of the biological product. It may be necessary for the manufacturer to file the proposed changes prior to implementation (21 CFR 601.12 and 21 CFR 610.9).

GLOSSARY

Acceptance criteria: The product specifications and acceptance or rejection criteria, with an associated sampling plan, necessary for making a decision to accept or reject a lot or batch (or any other convenient subgroups of manufactured units).  
active ingredient: Any component intended to furnish pharmacological activity or other direct effect in the diagnosis, cure, mitigation, treatment, or prevention of disease, or to affect the structure or any function of the body of man or other animals. The term includes those components that may undergo chemical change in the manufacture of the drug product and may be present in the drug product in a modified form intended to furnish the specified activity or effect.  
adventitious agent: A microorganism (e.g., bacteria, fungi, mycoplasma, spiroplasma, mycobacteria, rickettsia, viruses, protozoa, parasites, TSE agent) that is inadvertently introduced into the production of a biological product.  
batch: A specific quantity of a drug or other material intended to have uniform character and quality, within specified limits, and produced according to a single manufacturing order during the same cycle of manufacture.  
biological product: Any virus, therapeutic serum, toxin, antitoxin, or analogous product applicable to the prevention, treatment or cure of diseases or injuries of man.  
cell bank: Vials of cells of uniform composition (not necessarily clonal) derived from a single tissue or cell, aliquoted into appropriate storage containers, and stored under appropriate conditions.  
cell line: Cells that have been propagated in culture since establishment of a primary culture and have survived through crisis and senescence. Such surviving cells are immortal and will not senesce. Diploid cell strains have been established from primary cultures and expanded into cell banks, but have not passed through crisis and are not immortal.  
characterization: Determination of the properties of a substance.  
component: Any ingredient intended for use in the manufacture of a drug product, including those that may not appear in such drug product.  
container (also final container): The immediate unit, bottle, vial, ampule, tube, or other receptacle containing the product as distributed for sale, barter, or exchange.  
control: Having responsibility for maintaining the continued safety, purity, and potency of the product and for compliance with applicable product and establishment standards, and for compliance with current good manufacturing practices.  
control cells: Cells that are split off from the production culture and maintained in parallel under the same conditions and using the same reagents (e.g., culture medium) to perform quality control tests on cells that have not been exposed to the vaccine virus (which may interfere with some tests).
Dating period: The period beyond which the product cannot be expected beyond reasonable doubt to yield its specific results.

Diploid: Having the expected number of chromosomes for a species (i.e., two of each autosomal chromosome and two sex chromosomes).

Drug product: A finished dosage form (e.g., solution, suspension) that contains an active drug ingredient generally in association with inactive ingredients.

End-of-production cells: Cells harvested at the end of a production run or cells cultured from the master cell bank or working cell bank to a passage level or population doubling level comparable to or beyond the highest level reached in production.

End-of-production passage level: The maximal passage level achieved during manufacturing at final vaccine harvest. Cells may be evaluated at this level or beyond.

Endogenous virus: A virus whose genome is present in an integrated form in a cell substrate by heredity. Endogenous viral sequences may or may not encode for an intact or infectious virus.

Expiration date: The calendar month and year, and where applicable, the day and hour, that the dating period ends.

Filling: A group of final containers identical in all respects, which have been filled with the same product from the same bulk lot without any change that will affect the integrity of the filling assembly.

Final bulk: The stage of vaccine production directly prior to filling of individual vials.

Free of and freedom from: For a substance to be considered free of a contaminant, an assay must demonstrate that a defined quantity of the substance is negative for that contaminant to a defined level of sensitivity. The level of assay sensitivity is defined by the choice of assay and can be determined experimentally using standardized reagents. Alternatively, a validated process that is known to remove a contaminant to a defined level may be used to demonstrate freedom from that contaminant.

Harvest: Collection of material at the end of vaccine virus propagation in cell culture, from which vaccine will be prepared. This material may be the culture supernatant, the cells themselves (often in disrupted form), or some combination thereof.

Inactive ingredient: Any component other than an active ingredient.

In-process material: Any material fabricated, compounded, blended, or derived by chemical reaction that is produced for, and used in, the preparation of the drug product.

Intermediates: Unformulated active ingredients that are processed before final formulation and can be stored for long periods of time before further processing.

Label: Any written, printed, or graphic matter on the container or package or any such matter clearly visible through the immediate carton, receptacle, or wrapper.

Latent virus: A virus that is present in a cell, without evidence of active replication, but with the potential to reactivate, is considered to be microbiologically latent.

Lot: A batch, or a specific identified portion of a batch, having uniform character and quality within specified limits; or, in the case of a drug product produced by continuous process, it is a specific identified amount produced in a unit of time or quantity in a manner that assures its having uniform character and quality within specified limits.

Lot number, control number, or batch number: Any distinctive combination of letters, numbers, or symbols, or any combination of them, from which the complete history of the manufacture, processing, packing, holding, and distribution of a batch or lot of drug product or other material can be determined.

Manufacturer: All steps in the propagation or manufacture and preparation of products. Includes, but is not limited to, filling, testing, labeling, packaging, quality control, and storage by the manufacturer.

Manufacturer: Any legal person or entity engaged in the manufacture of a product subject to license under the Public Health Service (PHS) Act. Manufacturer also includes any legal person or entity who is an applicant for a license where the applicant assumes responsibility for compliance with the applicable product and establishment standards.

Master cell bank: A bank of a cell substrate from which all subsequent cell banks used for vaccine production will be derived. The master cell bank represents a characterized collection of cells derived from a single tissue or cell.

Master virus seed: A viral seed of a selected vaccine virus from which all future vaccine production will be derived, either directly, or via working virus seeds.

Oncogenicity: The property of certain biological agents (e.g., viruses) or materials (e.g., nucleic acids) that are capable of immortalizing cells and endowing them with the capacity to form tumors. Oncogenicity is distinct from tumorigenicity.

Package: The immediate carton, receptacle, or wrapper, including all labeling matter therein and thereon, and the contents of the one or more enclosed containers. If no package is used, the container shall be deemed to be the package.

Passage level: The number of times, since establishment from a primary cell culture, a culture has been split or reseeded.

Population doubling level: The number of times, since establishment from a primary cell culture, a culture has doubled in number of cells.

Potency: The therapeutic activity of the drug product as indicated by appropriate laboratory tests or by adequately developed and controlled clinical data. Potency may be expressed in terms of units by reference to a standard.

Primary cells: Cells placed into culture immediately after an embryo, tissue, or organ is removed from an animal or human and homogenized, minced, or otherwise separated into a suspension of cells. Primary cells may be maintained in medium, but are not passaged (split).

Process: A manufacturing step that is performed on the product itself which may affect its safety, purity, or potency, in contrast to such manufacturing steps which do not affect intrinsically the safety, purity, or potency of the product.

Proper name: The name, designated in the license, to be used on each package of the product.

Purity: Relative freedom from extraneous matter in the finished product, whether or not harmful to the recipient or deleterious to the product. Purity includes but is not limited to relative freedom from residual moisture or other volatile substances and pyrogenic substances.

Qualification: Determination of the suitability of a material for manufacturing based on its characterization.

Safety: The relative freedom from harmful effect to persons affected, directly or indirectly, by a product when prudently administered, taking into consideration the character of the product in relation to the condition of the recipient at the time.
**Specification:** The quality standard (i.e., tests, analytical procedures, acceptance criteria) provided in an approved application to confirm the quality of products, intermediates, raw materials, reagents, components, in-process materials, container–closure systems, and other materials used in the production of a product.

**Standards:** Specifications and procedures applicable to an establishment or to the manufacture or release of products, which are prescribed in this subchapter or established in the biologics license application and designed to ensure the continued safety, purity, and potency of such products.

**Sterility:** Freedom from viable contaminating microorganisms, as determined by tests prescribed by the FDA.

**Tumorigenic:** A property of certain cell types to form tumors when inoculated into animals (generally a syngeneic, an immunosuppressed allogeneic, or an immunosuppressed xenogeneic host). These tumors may be at the injection site or a different site and may also metastasize to other sites.

**Tumorigenicity:** The process by which immortalized cells form tumors when inoculated into animals. Tumorigenicity is distinct from oncogenicity.

**Unacceptable product:** Product that is no longer acceptable for use in clinical studies or for commercial use (e.g., because of degradation or loss of potency).

**Validation:** The performance characteristics of an analytical procedure, based on the demonstration that the procedure is suitable for its intended purpose or use. Validation of a process is the determination of the extent to which a process meets the requirements for the various performance characteristics and the demonstration that the process uniformly performs to defined characteristics. Validation is generally performed in accordance with *Validation of Compendial Procedures* (1225) and the relevant ICH guidelines.

**Viral clearance:** The combination of the physical removal of viral particles and the reduction of viral infectivity through inactivation.

**Virus seed or viral seed:** A live viral preparation of uniform composition (not necessarily clonal) derived from a single culture process, aliquoted into appropriate storage containers, and stored under appropriate conditions.

**Working cell bank:** A cell bank derived by propagation of cells from the master cell bank under defined conditions and used to initiate production cell cultures on a lot-by-lot basis.

**Working virus seed:** A viral seed derived by propagation of virus from the master virus seed under defined conditions and used to initiate production cell cultures lot-by-lot.

### APPENDICES

#### Appendix 1: Types of Vaccines Currently Licensed in the U.S. (examples)

- Bacterial, live attenuated (e.g., *Salmonella typhi*)
- Bacterial, polysaccharide (e.g., meningococcal, pneumococcal)
- Bacterial, polysaccharide-protein conjugate (e.g., meningococcal, pneumococcal)
- Bacterial, toxoid (e.g., diphtheria, tetanus)
- Bacterial, extracts (e.g., pertussis, anthrax)
- Viral, live attenuated (e.g., influenza, measles, mumps, rubella)
- Viral, whole inactivated (e.g., rabies)
- Viral, subunit (e.g., influenza, hepatitis B, human papillomavirus)

#### Appendix 2: Selected Regulatory Documents

- 21 CFR 201.
- 21 CFR 299.
- 21 CFR 600.
- 21 CFR 610.
- Section 262 in Title 42 of the Public Health Service Act
- FDA periodically issues or updates Guidance for Industry and posts these documents at [http://www.fda.gov/cber/guidelines.htm](http://www.fda.gov/cber/guidelines.htm)
〈1237〉 VIROLOGY TEST METHODS

INTRODUCTION

This chapter describes virology test methods applicable to the development of biological product drugs, such as recombinant proteins, subunit vaccines, therapeutic monoclonal antibodies, and growth hormones. Several topics are excluded from the scope of this chapter:

• Blood- and plasma-derived products as well as whole blood and plasma products used directly in transplantation or infusion. However, the basic principles, strategies, and testing methods for ensuring virus-free products are applicable.

• Methodologies for the safety testing of live viral vaccines.

• Specific methods for viral clearance studies, which are described in the USP general information chapter Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin (1050).

Virology test methods have historically been employed in the clinical settings of disease diagnosis, intervention, and containment; but the development of biological (biologics and biotechnology-derived) products and therapies for human or animal use has created the need for sensitive viral detection assays for use in the GMP production and testing of biological products. This need is not limited to the production of viral vaccines, but also applies to the development and manufacture of recombinant proteins, cell and gene therapies, and other products.

Sensitive virology test methods for quality control of biological products are necessary for several reasons. The production of biological products often requires a variety of raw materials and processing reagents of animal origin that have varying potential for introducing viral contaminants. The production of biological products may allow the replication of adventitious agents during processing, and therefore these materials must be prescreened to avoid the opportunity for contamination of the product. Another point to consider regarding screening these materials is that the product may not be compatible with processing methods used to eliminate or inactivate these adventitious agents. Because of the nature of the biological products, the production process needs to include appropriate testing regimens that monitor the possible introduction of adventitious agents and/or viral agents into the systems used. For these reasons, sensitive viral detection methods are required not only for the release testing of biological drug products, but also during the intermediate stages of processing, process development, and routine manufacture. Important stages for consideration include the development of cell substrates and banks, raw materials of animal origin, process intermediates, and critical excipients when derived from animal tissues. This strategy should be augmented with viral clearance and inactivation studies whenever possible.

For products intended to contain live viruses (e.g., infectious oncolytic viruses and live viral vector products used for gene therapy), the cell- and animal-based infectivity methods discussed in this chapter may be useful only following neutralization of the specific viral entity contained in the product for any product that is intended to contain live viruses (e.g., infectious oncolytic viruses and live viral vector products used for gene therapy). Alternatively, selection of appropriate indicator cell lines or animal models in which the specific viral entity is known not to replicate can be considered. It should also be expected that assay systems based on detection of viral particles or viral components will indicate the presence of the viral entity itself in such products, but may not indicate the viability of the virus. The remainder of the chapter is divided into three sections discussing assays for the three topics: (1) Detection of Viable Viruses, (2) Detection of Viral Components, and (3) Detection of Antibodies to Viral Antigens. The chapter covers the classic virology methods that are still routinely used, as well as modern molecular and immunological approaches. The methods described in these sections may possess different sensitivities to diverse viruses; they are therefore intended to complement each other to provide a science-based foundation for the detection of adventitious viruses. Multiple methods may be used in complementary fashion to improve the pathogen safety margin of a product. Identification of viruses detected in cell-based assays on the basis of cytopathic effects often depends on the use of molecular and immunological analyses; these analyses are therefore relevant both to viral detection and to subsequent viral identification. The chapter provides an overview of the detection and analysis of the most important groups of viruses as well as the most commonly used techniques. Tests specific to individual vaccines or biological products are excluded, because they are expected to be included in monographs for such products.

Methods that are well established with little variation in practice are described in more detail, whereas methods that are more flexible are described in general terms, both in the performance of the tests and in considerations for acceptance. Relevant regulatory references are given in the Appendix. Relevant USP general chapters should be consulted with regard to bioassay design, data analysis, interpretation, and assay validation.

DETECTION OF VIABLE VIRUSES

Infectious virus particles contaminating biologics and biotechnology-derived products are of great safety concern, because they have the potential for causing serious, possibly life-threatening, infections in the patients treated. This is particularly true if the patients are immunocompromised. Although complete assurance of viral safety for finished biological products can never be realized, a significant safety margin can be established through viral detection methods applied to unprocessed bulk and raw materials before purification in combination with purification processes that demonstrate the ability to inactivate or remove potential viral contaminants present at levels too low to detect. (See Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin (1050) for information on viral clearance and inactivation methods.) For cell and gene therapy products that lack extensive purification steps, final products may be directly tested for the presence of relevant contaminating viruses. This section describes two broad systems for the detection of infectious virus: cell culture-based infectivity assays and in vivo infectivity assays. These systems may possess complementary sensitivities for viruses, and as a result, both methods may be used as limit tests for cell bank and raw material characterization and for lot release testing of biologics and biotechnology-derived products. Considerations for optimizing sample preparation for these tests are discussed, followed
Sample Selection of and Preparation for Cell- and Animal-Based Virus Detection Assays

The requirements for selection, preparation, and storage of test samples for viral detection methods (cell- and animal-based) are dictated by the lability of the viruses being detected. The ability of a virus to remain infectious in the absence of a host cell is highly variable. Virus infectivity also may differ in sensitivity to repeated freezing and thawing cycles.

Sample preparation typically involves storage of test samples at low temperatures (ideally −60° or below) as soon as practicable upon collection. When intended for use in a viral screening assay, aliquots of samples should be prepared to avoid multiple freezing and thawing. Samples intended for viral infectivity assays are typically shipped with sufficient dry ice to last several days more than the expected time required for transit. When received at the testing laboratory, the sample should be examined to verify that it is still frozen, and appropriate documentation should be completed. For any storage or hold condition, the impact of the condition on viral viability should be empirically assessed and sufficient cold chain management ensured.

Typical sample types for viral detection assays are described below.

CELL LYSATES

Test samples derived from cell substrates (master and working cell banks, end-of-production cell samples) are prepared in a manner that allows sampling of both the cells (for cell-associated viruses) and the conditioned medium (for virus shed into the medium). To achieve this, a culture of the cells is sampled. A cell suspension of ~10^7 cells per mL in conditioned medium is prepared and frozen (ideally at −60° or below). Because this medium does not contain cryopreservative, the majority of the cells will lyse upon thawing of the sample, releasing the cell-associated virus. Low-speed centrifugation will remove larger cellular debris and yield a supernatant that may be inoculated directly onto detector cells in cell-based viral infectivity assays. A similar sample is prepared for in vivo viral adventitious agent testing. In this case, however, the test sample is thawed and injected without clarification into the various animal systems via the various described routes.

BIOTECHNOLOGY BULK HARVEST (UNPROCESSED BULK HARVEST) SAMPLES

Routine lot testing of bulk harvest samples is mandatory for most types of biologics (see Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin (1050)). The sampling must be done at the unprocessed bulk harvest stage, because downstream purification processes may remove or inactivate any viruses that might contaminate the starting materials. The harvest sample from the bioreactor should be collected and stored without further manipulation, as soon as practicable, at −60° or below. To prevent multiple freeze and thaw cycles, individual aliquots should be prepared for each individual assay to be performed. Additional aliquots should be retained in case repeat testing is required. Depending on the nature of the manufacturing process, the bulk harvest samples may contain varying quantities of the production substrate cells. Because the bulk harvest does not contain cryopreservatives, the majority of the cells present will lyse upon thawing of the sample, releasing the cell-associated virus. Low-speed centrifugation to clarify the sample will result in a supernatant that may be inoculated directly onto detector cells in cell-based viral infectivity assays. There may be instances where the test sample is cytotoxic to the detector cells of the cell-based assays and procedural modifications may be required to deal with this. A similar sample is prepared for in vivo viral safety testing. In this case, however, the test sample is thawed and injected without clarification into the various animal systems via the various described routes.

RAW MATERIALS OF ANIMAL ORIGIN

Ingredients of animal origin used in the manufacture of biological products for human or veterinary use must be tested for species-specific viruses of concern as described in 9 CFR 113.53 (see also the USP general information chapter Bovine Serum (1024), being prepared for future publication). The raw materials may be stored under a variety of conditions, as appropriate to the raw material. Sample preparation and method of application to the test system depend on the nature of the sample. The possibility that animal-derived raw materials may contain bacterial or fungal contaminants should be considered. In some cases, it may be necessary to treat the samples with antibiotics or to filter the samples (0.22 or 0.45 micron pore size) prior to inoculation in order to prevent bacterial or fungal outgrowth in the test system. Animal sera are typically received frozen and are thawed and incorporated into the growth medium at an appropriate concentration (typically 15%, v/v) as a means of exposing the detector cells. Powdered trypsin (not less than 5 grams, as per 9 CFR 113.53) is suspended in a suitable diluent, such as phosphate-buffered saline, and is then subjected to high-speed centrifugation to pellet any virions that may be present. The concentrated pellet is resuspended in phosphate-buffered saline, and the resulting material is used to inoculate appropriate detector cells. Medium additives, such as bovine thrombin, may be incorporated into the growth medium at a predetermined multiple of the nominal concentration to be used in the manufacturing process. The resulting growth medium containing the additives is then used as a means of exposing the detector cells to the test material. The exact multiple to be used in such testing may be limited by such factors as solubility in growth medium or cytototoxicity to the detector cells. These factors should be assessed in advance of testing. The principle of using higher concentrations in the detection method than during processing should be followed, within the bounds of indicator cell toxicity, as a means to increase sensitivity to detection.

WHOLE CELLS

Intact viable cells are used as the test sample in certain viral detection assays. Because the test cells may attach and proliferate in the culture vessel along with the detector cells, assays using this type of sample are referred to as cocultivation assays. The requirements for the specific assay may vary in relative proportions of detector and test cells, viability of the test cells, or the confluency of test cells at the time of collection.
Cell Culture-Based Viral Detection Methods

To ensure the absence of adventitious viral agents, cell culture–based viral detection assays are used for a variety of purposes, including but not limited to clinical diagnostic procedures; evaluation of raw materials and cell substrates; assessments of the viral identity, the purity, and the potency of virus seed stocks; and lot release testing of unprocessed bulk harvests during biologics production. An important distinction between cell-based assays and direct detection assays (see the section Detection of Viral Components) is that the former will detect only replicating virus, whereas the latter will detect viral antigens, viral genomic material, and the like, which may or may not be indicative of the presence of replicating virus. Similarly, detection of circulating antibodies directed against viral antigens (discussed in the section Detection of Antibodies to Viral Antigens), may be indicative of either a current or a past infection of an animal and does not necessarily indicate that the animal is currently harboring an infection.

Infectious viruses detected in cells or in cell-derived materials fall into two broad categories, based on the expectations of the analyst. Endogenous viruses are those normally detected in the cells as a result of the integration of the viral genomic material into the host cell DNA. Exogenous viruses are those not normally present in the cells but found as a result of a viral infection of the cells.

The underlying assumption for all cell-based viral detection methods is the ability of viruses to replicate in an appropriate host cell. Viruses lack the cellular machinery required for producing their own genomic material and structural proteins, and they must therefore enter and subordinate a host cell for this purpose. Cell-based viral infectivity assays use indicator (detector) cells that serve as host cells for viable virions present in test samples.

Cell-based infectivity assays may be placed in three broad categories on the basis of types of viruses to be detected: (1) retroviral assays, (2) virus-specific assays, and (3) viral screening assays. The types of endpoints used to detect the viruses may differ by category. Although screening assays are typically not optimized for single viral entities, the virus-specific assays and titration assays, as well as some of the retroviral assays, may be optimized to some extent for specific viruses. Accurate titration of stock viruses that are used as positive controls or are used to determine the detection limit of an assay is critical.

The regulatory guidance underlying the various viral safety tests depends on the nature of the samples to be evaluated, and analysts are referred for more detail to documentation relevant to their own regulatory environments.

General Requirements for Cell Culture–Based Assays

DETECTOR CELLS AND THE CONCEPT OF VIRAL HOST RANGE

The range of viruses detectable using a cell-based infectivity assay depends on a number of factors, including the type of host cell(s) used as the indicator (detector) cultures and the detection endpoints used in the assay. Viruses differ in their abilities to infect specific host cell types. Most viruses exhibit at least some degree of host cell tropism (i.e., ability to infect a specific species or tissue type). This attribute is typically due to a requirement for interaction of a virion with a specific cell membrane receptor during the process of infection of the host cell. A cell susceptible to infection and capable of production of progeny by a given virus is referred to as permissive for that virus; cells not supporting viral proliferation are referred to as nonpermissive, or restricted, for that virus. As a consequence of the differences in host cell tropism, assays intended to screen for a wide range of viruses must include multiple detector cell types. For the same reasons, design of a cell-based infectivity assay for a specific virus must include a detector cell known to be permissive for that virus.

VIRUS SUSCEPTIBILITY OF COMMON CELL LINES

For most endpoint assays used to determine whether a host cell is infected with a virus, a monolayer culture is preferable to a semiadherent or suspension culture. For instance, cytopathic effect and hemadsorption are visualized microscopically. Cells that are not adherent have little morphology to evaluate, and hemadsorption cannot be properly evaluated in a suspension culture. For this reason, some regulatory documents pertaining to cell-based virus infectivity assays stipulate the use of monolayer detector cultures.

A list of commonly employed indicator cell lines and their application in viral screening assays is provided in Table 1. Regarding the viral tropism of these cells, “Points to Consider in the Characterization of Cell Lines Used to Produce Biologicals” (1993) and ICH’s Q5A (R1) guidelines (for these references, see Appendix) require that human diploid cells such as MRC-5 and WI-38, which are permissive for a range of viruses of human concern, and monolayer cultures of the same species as that of the cell substrate used to produce the product are included in the viral screening test for biologics destined for use in humans.

### Table 1. Indicator (Detector) Cell Lines Used for Adventitious Viral Screening Assays

<table>
<thead>
<tr>
<th>Cell Line*</th>
<th>Origin</th>
<th>Endpoint(s)*</th>
<th>Target virus(es)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BHK-21</td>
<td>Syrian hamster</td>
<td>CPE, HA, HA</td>
<td>Insect-borne viruses (arboviruses)</td>
</tr>
<tr>
<td>Vero</td>
<td>African green monkey</td>
<td>CPE, HA, HA</td>
<td>Viruses infectious to humans, primates</td>
</tr>
<tr>
<td>HeLa</td>
<td>Human</td>
<td>CPE, HA, HA</td>
<td>Viruses infectious to humans</td>
</tr>
<tr>
<td>MRC-5</td>
<td>Human</td>
<td>CPE, HA, HA</td>
<td>Viruses infectious to humans</td>
</tr>
<tr>
<td>CHO-KI</td>
<td>Chinese hamster</td>
<td>CPE, HA, HA</td>
<td>Viruses infectious to Chinese hamsters</td>
</tr>
</tbody>
</table>

*Cell lines with relatively broad viral tropism:

For processes involving human cell substrates:

For processes involving Chinese hamster cell substrates:
Table 1. Indicator (Detector) Cell Lines Used for Adventitious Viral Screening Assays (continued)

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Origin</th>
<th>Endpoint(s)</th>
<th>Target virus(es)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEF</td>
<td>Mouse</td>
<td>CPE, HA, HA</td>
<td>Viruses infectious to mouse cells</td>
</tr>
<tr>
<td>NIH/3T3</td>
<td>Mouse</td>
<td>CPE, HA, HA</td>
<td>Viruses infectious to mouse cells</td>
</tr>
<tr>
<td>For processes involving bovine cell substrates or bovine raw materials:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDBK</td>
<td>Bovine</td>
<td>CPE, HA, HA</td>
<td>Bovine viruses</td>
</tr>
<tr>
<td>BT</td>
<td>Bovine</td>
<td>CPE, HA, HA</td>
<td>Bovine viruses</td>
</tr>
<tr>
<td>EBTr</td>
<td>Bovine</td>
<td>CPE, HA, HA</td>
<td>Bovine viruses</td>
</tr>
</tbody>
</table>

a Examples of cell lines used for viral screening assays are shown. MRC-5 and Vero, or cells with similar host ranges, are used in all assays. Depending on the cell substrate used to manufacture a biologic, additional cell lines are also used in the screening assay. In addition, a bovine cell might be included if bovine serum was used in the manufacturing process.

b CPE, cytopathic effect; HA, hemadsorption; HA, hemagglutination (optional).

c Inclusion of a bovine cell in a virus screen should not be construed as a replacement for or alternative to a raw materials test. Raw materials testing is driven in the U.S. by 9 CFR 113.47 and 113.52, and cell lines used for this testing are described in Table 2.

A list of commonly employed indicator cell lines and their application in raw materials testing assays is provided in Table 2.

Table 2. Indicator (Detector) Cell Lines Used in Raw Material Testing

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Assay Type</th>
<th>Endpoint(s)</th>
<th>Animal Origin of Raw Material</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vero</td>
<td>Isolation/detection</td>
<td>CPE, HA, IFA</td>
<td>All sources</td>
</tr>
<tr>
<td>BT</td>
<td>Isolation/detection</td>
<td>CPE, HA, IFA</td>
<td>Bovine; all sources (BVDV)</td>
</tr>
<tr>
<td>EBTr</td>
<td>Isolation/detection</td>
<td>CPE, HA, IFA</td>
<td>Bovine; all sources (BVDV)</td>
</tr>
<tr>
<td>MDBK</td>
<td>Isolation/detection</td>
<td>CPE, HA, IFA</td>
<td>Bovine; all sources (BVDV)</td>
</tr>
<tr>
<td>PT-1</td>
<td>Isolation/detection</td>
<td>CPE, HA, IFA</td>
<td>Porcine</td>
</tr>
<tr>
<td>PK-1</td>
<td>Isolation/detection</td>
<td>CPE, HA, IFA</td>
<td>Porcine</td>
</tr>
<tr>
<td>MDCK</td>
<td>Isolation/detection</td>
<td>CPE, HA, IFA</td>
<td>Canine</td>
</tr>
<tr>
<td>GT</td>
<td>Isolation/detection</td>
<td>CPE, HA, IFA</td>
<td>Caprine</td>
</tr>
</tbody>
</table>

The requirement (9 CFR 113.47 and 113.52) for evaluating raw materials of animal origin is to use (1) Vero cells, (2) a bovine cell for detecting BVDV, and (3) a cell line of the same species of origin as the raw material for detecting viruses of concern from that species. Examples are given of some cell lines that are used in the industry.

b CPE, cytopathic effect; HA, hemadsorption; IFA, immunofluorescent antibody staining.

c As per 9 CFR 113.47, raw materials of any animal origin are to be tested for bovine viral diarrhea virus (BVDV).

There may be very specific requirements for detector cells for certain viruses. For instance, assays intended to detect infectious HIV use human peripheral blood lymphocytes and involve a p24 antigen capture enzyme immunoassay endpoint. A list of commonly employed indicator cell lines and their application in the detection of specific viruses is provided in Table 3.

Table 3. Indicator (Detector) Cell Lines Used for Detection of Specific Virus(es)

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Assay Type</th>
<th>Endpoint(s)</th>
<th>Target Virus</th>
</tr>
</thead>
<tbody>
<tr>
<td>324K</td>
<td>Isolation/detection</td>
<td>CPE, HA, IFA</td>
<td>Murine minute virus</td>
</tr>
<tr>
<td>A</td>
<td>Isolation/detection</td>
<td>CPE, HA, IFA</td>
<td>Murine minute virus</td>
</tr>
<tr>
<td>BHK-21</td>
<td>Isolation/detection</td>
<td>CPE, HA</td>
<td>Arboviruses</td>
</tr>
<tr>
<td>MRC-55</td>
<td>Isolation/detection</td>
<td>CPE</td>
<td>Human cytomegalovirus</td>
</tr>
</tbody>
</table>

a Examples of cell lines used for optimizing the detection of specific viruses or virus types are shown. In many cases, the assay methodologies must also be optimized for detection of the target viruses.

b CPE, cytopathic effect; HA, hemadsorption; IFA, immunofluorescent antibody staining.

c Insect-borne viruses as a group are referred to as arboviruses. This term has no taxonomic significance.

d Other human diploid cell lines such as WI-38 are also suitable. Assay duration must be 28 days at a minimum.

GROWTH REQUIREMENTS FOR DETECTOR CELLS

Viral proliferation within a permissive host cell may be dependent on the rate of host cell proliferation. This is especially true for viruses that display cell-cycle dependence for generation of viral progeny. For most detection assays, detector cultures are seeded at a density intended to achieve a cell monolayer in exponential growth. This corresponds to a cell confluence of 50% or less (optimal cell densities may depend on the assay type and the detector cell to be used) at the time of inoculation of the cultures with virus or test sample. For the same reasons, the assay design may include provision for detector cell subculture (the collection of cells from the original culture and seeding of a predetermined fraction of these into a new flask). Alternatively, a
passage may be performed, consisting of collection of conditioned medium from the original culture and inoculation of this material onto a secondary detector cell culture that is in log-phase growth. The frequency of subculture/passage required in an assay is determined largely by the rate of growth of the detector cell. Incorporation of these steps into detection assay designs helps to ensure that the conditions remain optimal for amplification of viral progeny within the host cells.

NEED FOR DETECTOR CELL IDENTIFICATION AND BANKING

Detector cells used for cell-based viral detection assays are a critical reagent for ensuring viral safety, viral potency, viral identity, and viral clearance capacity in purification schemes. In many cases this testing is intended to support GMP processes; therefore, the detector cell banks may need to be prepared and qualified in much the same manner as other critical reagents. The details of the viral safety evaluation methods are described in *Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin* (1050). Regardless of specific compliance requirements, periodic identification and qualification of the detector cell banks to be used subsequently for viral infectivity assays is good practice. The following should be regarded as minimal quality control testing for such detector cell banks: sterility, mycoplasma and viral screening, and cell identity by DNA fingerprinting, karyology, or isoenzyme analysis. In addition, specific assays for bovine and porcine viruses may be required if bovine or porcine raw materials were used in preparation of the banks. *Bovine Serum* (1024) and relevant serum-type specific ancillary materials monographs should be consulted when serum products are used in cell growth media.

Endpoints for Detection of Viral Infection

The various endpoints used to identify infection of a detector cell with a virus include the following:

- Visual observation of cytopathic effects
- Hemagglutination or hemadsorption of erythrocytes
- Immunofluorescent staining
- Cocultivation with other types of detector cells
- Quantitative polymerase chain reaction (qPCR) for direct detection of viral genomic sequences
- Electron microscopic analysis of viral pellets or fixed cells for visual observation of viral particles
- Biochemical endpoints such as reverse transcriptase assays, which detect virus-specific enzymatic activity

These various endpoints are used in a complementary fashion, because a given virus may not cause a positive response in each endpoint. For instance, some viruses can grow to high titers without producing visible cytopathic effects and so must be detected using other endpoints. Polymerase chain reaction (PCR) and electron microscopic analysis per se are not capable of distinguishing viable from nonviable viruses. However, when used in conjunction with cell culture growth kinetics, these approaches can be powerful orthogonal detection methods to demonstrate the increase of viral replication and therefore viable virus. The failure to observe viral particles in electron microscopic analysis of fixed cells should not be considered absolute proof of the absence of infectious virus in the cells. In a general sense, the same is true for each of the detection endpoints discussed above. Each endpoint has a detection limit below which a virus may be present but not detected.

VIRAL CYTOPATHIC EFFECTS

Visually observable manifestations of the infection of susceptible host cells with certain types of viruses are collectively referred to as viral cytopathic effects (CPE). Although CPE may be considered an indirect detection of viral infection, in the context of specific host cells they can have distinctive morphological manifestations. These may include the appearance of inclusion bodies, abnormal cell morphology, changes in culture confluency, cell death and cell lysis, and others. The nature of the CPE observed may depend on the host cell and the infecting virus. In addition, for a virus that normally causes CPE, there may exist variants that do not cause CPE. CPE can be differentiated from cytotoxic effect by the tendency of the former to exhibit progression irreversibly with time, whereas the latter may be reversible. In some cases structural proteins of viruses may cause cytotoxic effects similar to the cytopathic effects of the infectious virus. Differentiating the cytotoxic effect of such proteins from the cytopathic effect of infectious virus may require observation of the culture over time to determine whether the effect progresses or the cells appear to recover. Alternatively, cell-free passage of the original culture onto fresh detector cells can be used to differentiate these two apparently similar manifestations. The cytotoxicity associated with the structural proteins in the absence of infectious virus would not be expected to pass to the secondary culture.

Giemsa staining may optimize the ability of the operators to visualize certain inclusion bodies (clusters of viral particles) that are characteristic of viral cytopathic effect and is required by 9 CFR 113.53 in assays used to demonstrate that bovine, porcine, equine, and ovine raw materials are free of species-specific viruses.

DETECTION OF HEMAGGLUTINATING VIRUSES

A characteristic of certain viruses (referred to as hemagglutinating viruses) is that one or more of their viral proteins cause hemagglutination of one or more types of erythrocytes. Hemagglutination is an interaction between viral proteins or hemagglutinins and erythrocytes, leading to adhesion of the erythrocytes to surfaces, cells, and each other. This property forms the basis of two endpoint procedures that are employed in cell-based viral infectivity assays: hemadsorption and hemagglutination.

Hemadsorption is performed by adding a suspension of one or more erythrocyte types directly to the monolayer culture of detector cells. If viral proteins of a hemagglutinating virus are expressed from infected cell membranes, the susceptible erythrocytes will bind tightly to the cell membranes. Noninfected cells do not display this binding; therefore, the technique can be used to visualize a focus of infected cells against a background of uninfected cells. For this reason, this particular endpoint may display greater detection sensitivity than other assay endpoints, such as cytopathic effect or hemagglutination. In the
advanced stages of infection, binding of erythrocytes to cells, to each other, and to open plastic surfaces in the culture vessel may be observed.

The hemagglutination procedure is performed on the conditioned medium and is essentially an evaluation for free virus or viral hemagglutinins in solution. An aliquot of the conditioned medium from a detector culture is combined, in a microwell plate having v-bottomed wells, with one or more types of erythrocytes. After an appropriate amount of time the plates are evaluated. Absence of hemagglutination is reflected by a well-defined pellet (button) of erythrocytes sedimenting to the bottom of the well. In comparison, hemagglutination is reflected by the absence of a button, or by a button with irregular shape. Scoring the latter represents an opportunity for operator subjectivity. In addition, this endpoint can be considered the least sensitive in detection assays, because it is dependent on achieving a sufficient concentration of viral hemagglutinins in solution. For these reasons, hemadsorption is typically viewed as the more useful and reliable of the techniques for detecting hemagglutinating viruses.

The responses obtained in detection assays using hemadsorption and hemagglutination endpoints are highly dependent on the virus being assayed, as well as on the types of erythrocytes used. Many of the viruses of concern to the biotechnology industry do not cause hemadsorption and hemagglutination, or their hemagglutinins react with red blood cell types not commonly used in detection assays.

**DETECTION BY IMMUNOFLUORESCENT ANTIBODY (IFA) STAINING**

Certain cell-based viral detection assays are intended to detect specific viral entities and are required for raw materials tests derived from bovine-, porcine-, equine-, and ovine-derived materials (9 CFR 113-53). In order to achieve this, the assays must be optimized with respect to host cell selection, study design, and sample preparation. Specificity of detection is also conferred through use of IFA staining techniques. Primary antisera or monoclonal antibodies directed against the viral antigens of interest are used, either in direct staining applications or in conjunction with a fluorochrome-conjugated secondary antiserum. The immunostained detector cell monolayers are then visualized with an epifluorescence microscope to reveal the presence of reactive infected cells.

**Design of Cell-Based Viral Assays**

**Viral Detection Assays**

Detector cell cultures are seeded and allowed to incubate for the appropriate amount of time. Viral samples may be inoculated directly into the medium of the mitotic phase of cell culture, but more typically the medium is removed from the overnight detector cell culture and is replaced with the test sample. For the latter method, the test sample must therefore be approximately isotonic, and cytotoxic agents such as selection agents must be maintained within levels tolerable to the detector cell. For test samples comprised of live cells, the sample must be subjected to freeze-thaw before inoculation in order to lyse the sample cells. If the cells are not lysed, a cocultivation involving the detector and sample cells will result. The latter is part of the design for cocultivation assays. But for most of the cell-based infectivity assays such a cocultivation is not intended and could adversely impact the sensitivity of the assay. The test sample is typically allowed to adsorb to the detector cell monolayers for an appropriate amount of time. It is then removed and replaced with the growth medium suitable for the detector cell. Once inoculated, the detection assay involves incubation of the detector cells for a prescribed amount of time, with periodic refeeding as necessary. Endpoint evaluations as described above are performed according to the study design. Variations of the given procedure may be used for exposing detector cells to raw materials. For raw materials, detector cell exposure may consist of incorporation of the test materials into the growth medium used to maintain the detector cells throughout the assay.

**Viral Titration Assays**

Viral titration assays are designed to generate quantitative information about the virus of interest. These assays do not quantify absolute numbers of viral particles; rather, the results are expressed in terms of infectious units. An infectious unit is the amount of virus required to establish a productive infection, and several categories of titration assays are used. They vary on the basis of the endpoint used to demonstrate infection and include viral plaque titration or plaque-forming units (units: PFU per mL); 50% tissue culture infectious dose (units: TCID₅₀ per mL); and 50% fluorescent antibody infectious dose (units: FAID₅₀ per mL). The amount of a hemagglutinating virus in a sample can also be expressed in terms of hemagglutinating titer (units: endpoint dilution, i.e., the greatest dilution of the sample which still results in a positive hemagglutination, or HA, response).

Regardless of the endpoint used, a typical titration assay design consists of a sequence of sample dilutions based on 0.5 log₁₀ or 1 log₁₀ increments. The various dilutions of the sample are then applied to an appropriate number of replicate permissive detector cell monolayers. After a suitable incubation time, the monolayers are scored directly for cytopathic effect (TCID₅₀ assay), fixed and processed for immunostaining and scored for reactive cells (FAID₅₀ assay), or overlaid with agarose and processed for plaque generation PFU assay. TCID₅₀ and FAID₅₀ titers are typically calculated using published formulas, such as Spearman-Kärber and Reed-Muench. Assay controls for such quantitative assessments should routinely include a reference sample of known potency.

**Detection of Retroviruses**

Retroviruses represent a special case for cell-based viral detection assay because of the occurrence of retroviral infection in the absence of responses to the typical endpoints discussed. In order to detect retroviruses, scientists can employ a number of different endpoints. A list of commonly employed indicator cell lines and associated endpoints, and their application in retrovirus detection assays, is provided in Table 4.
Retroviral infection is dependent on the presence of receptors on the host cell membranes. The presence of such receptors confers host cell tropism.

**DESIGN FOR RETROVIRUS INFECTIVITY ASSAYS**

Two types of infectivity assays are used, depending on the nature of the test material. For materials other than intact cells, the test material is inoculated onto one or more of a variety of detector cells, and the latter are then passaged as required to amplify any virus present. Because of the nature of retroviral replication, cytopathic effects typically do not occur during infection, although there are some exceptions. Before the first subculture and at the end of the final passage, one or more endpoint assays are employed to detect the presence of a retrovirus. For test materials in the form of intact cells, detector cells are seeded and subsequently inoculated with the test cells, resulting in a cocultivation. The cultures are passaged five or more times. Before the first subculture and at the end of the final passage, one or more endpoint assays are employed to detect the presence of a retrovirus.

**ENDPOINT ASSAYS FOR RETROVIRUS DETECTION**

Endpoint assays may be classified as direct, which lead to distinct morphological changes in the detector cells; or indirect, as measured by the detection of biochemical, molecular, or immunological markers for infection.

**XC-Plaque Assay**—The XC-plaque assay was developed as a direct means of detecting infectious murine retroviruses. Detection of the retrovirus is accomplished by UV-irradiating the detector cells used to amplify the virus and overlaying the irradiated detector cell cultures with a specific rat cell (XC). The presence of infectious murine retroviruses in the detector cells is reflected by the formation of distinctive syncytia in the XC monolayer, which are easily visualized when the cultures are fixed and stained with a suitable dye such as crystal violet.

The N/B tropism of an ecotropic murine virus may be determined by inoculating Balb/c and NIH Swiss detector cells with the isolate, performing one or two passages on each cell line, and comparing the XC-plaque titer post passage to that determined for the initial isolate.

**Mink and Feline S+L− Focus Assays**—The S+L− focus endpoint was developed to facilitate direct detection of infectious murine xenotropic and amphotropic viruses. The test sample may be inoculated directly into cultures of the S+L− cells, or, alternatively, may be amplified first by inoculation into mink lung, human, or Mus dunni detector cells. Cell-free supernatants from the detector cell cultures are used to inoculate the S+L− cells in the absence of helper leukemia virus to render it capable of causing transformation of the host cell. The presence of infectious retrovirus is reflected by the formation of characteristic focal areas of cell transformation in the S+L− cells caused by the rescued sarcoma virus.

**Detection of Retroviral Reverse Transcriptase**—Assays designed to measure reverse transcriptase (RT) activity are useful as an indirect detection method, because the enzyme is indicative of the presence of all retroviruses, whether infectious or not. The RT enzyme is encoded for in the retroviral genome and is used by the virus to transcribe genetic information in viral genomic RNA into proviral DNA.
Radionabeled Nucleotide Incorporation Assay—The earliest methods for measuring RT activity were based on the measurement of \(^{32}\text{P}\) or \(^{3}\text{H}\)-labeled nucleotide incorporation into the complementary cDNA product, using an appropriate RNA template. Incorporation of radiolabeled nucleotide at levels higher than a predetermined threshold is interpreted as evidence of the presence of retroviral RT activity. The contributions of cellular DNA polymerases can be ruled out through use of a dual template assay (having both RNA and DNA templates) or inclusion of activated calf thymus DNA.

Product-Enhanced Reverse Transcriptase (PERT) or Quantitative PERT (Q-PERT)—Polymerase chain reaction amplification has been used to increase the sensitivity of RT activity measurement. RT activity is detected by PCR amplification of complementary DNA, newly synthesized from an RNA template by reverse transcriptase. The assay may be performed with a gel endpoint (PERT) or as a quantitative assay (Q-PERT). This method has increasingly gained acceptance by regulatory agencies. For more details on PCR-based techniques, see the USP general information chapter Nucleic Acid Based Techniques—Amplification (1127).

**ELECTRON MICROSCOPY**

Transmission electron microscopy (TEM) may be used to detect and enumerate viral particles within cells. In addition, the technique allows for differentiation of types A, B, C, and D retroviruses based on morphological considerations and can be used to localize viral particles within the cell. As a technique for identification of viruses (including RNA and DNA viruses in general), TEM of sectioned cells is extremely valuable. The cells, typically sampled during the log phase of growth, are pelleted by low-speed centrifugation, and the cell pellet is fixed with a suitable fixative. The fixed cell pellet is embedded, sectioned, stained, and observed with TEM. Size (diameter) of the particles, morphology, presence or absence of surface features such as envelopes and spikes, and location within the cell can be determined with this technique. Such information is important for the identification of a virus. However, failure to observe viral particles with this method does not conclusively demonstrate the lack of viral contamination in the sample.

Biological fluids may also be evaluated by TEM, primarily to determine particle size and concentration. The cell-free supernatant is subjected to ultracentrifugation to pellet any virus present. The resulting pellet is fixed with a suitable fixative. A predetermined number of grid spaces containing representative areas of thin sections of the pellet are evaluated for particles. The enumeration results obtained may show a high degree of variability, and failure to observe particles does not imply that none were present in the sample. Molecular (quantitative PCR and quantitative PERT) endpoints have also been used as alternative methods for estimation of viral particle load in samples.

**ANTIGEN-CAPTURE ENZYME IMMUNOASSAY**

Specific viral proteins (e.g., HIV p24 antigen or avian leucosis viral envelope proteins) may be detected as a means of determining the presence of a retrovirus. Viral antigens are captured by specific antibodies coated onto microtiter plate wells and are detected by the addition of a second labeled antibody and appropriate substrate.

**Assays Designed to Detect Specific Viruses**

Additional methodologies have been developed to allow detection of specific viruses or groups of viruses. These types of assays are often used for raw material evaluation. In some cases, these specific assays were developed because the target viruses do not cause endpoint responses in the viral screening assays. In contrast to screening assays, specific virus assays are typically optimized for detection of the target virus or viruses. This optimization takes into account the lability of the virus, the host range, the possible endpoint responses elicited, and any special requirements of the target virus. The use of well-characterized viruses as positive controls in such assays provides assurance that the methodologies are suitable for the target virus or viruses. Spiking of the test sample matrix with the positive control virus enables the investigator to assess the potential for matrix interference and to assess the limit of detection for the method. Such considerations are not applicable to screening assays. Specific virus testing for bovine- and porcine-derived raw materials is discussed below. Evaluation of caprine, ovine, equine, canine, and feline raw materials is also stipulated in 9 CFR section 113.47. This section should be consulted with respect to the viruses of concern, and 9 CFR 113.52 should be consulted for methodology. A list of commonly employed indicator cell lines and their application in raw materials testing assays is provided in Table 2. A list of commonly employed indicator cell lines and their application in detection of specific viruses is also provided (see Table 3).

**DETECTION OF BOVINE VIRUS CONTAMINATION**

Raw materials of bovine origin include such commonly employed medium components as fetal bovine and calf serum, serum albumin, collagen, thrombin, and trypsin. Each of these additives represents a route of entry for adventitious viral contaminants into a cell culture or manufacturing process. Requirements for evaluation of such materials ensure the absence of contaminating viruses.

For the details on testing for bovine serum and its derivatives, see future general chapter Bovine Serum (1024).

**DETECTION OF PORCINE VIRAL CONTAMINANTS**

Raw materials of porcine origin include trypsin as well as other cell culture reagents. The specific porcine viruses of concern in the United States are stipulated in 9 CFR 113.47 and include porcine parvovirus, porcine adenovirus, transmissible gastroenteritis virus, and porcine hemagglutinating encephalitis virus. In addition, porcine raw materials must also be evaluated for the presence of bovine viral diarrhea virus (BVDV), reovirus, and rabies virus. Porcine tissues intended for xenotransplantation into humans also are routinely evaluated for the porcine endogenous retrovirus (PERV). The host cells typically used in the detection of porcine viruses are porcine testicle or porcine kidney, a bovine cell, and Vero cells. The methodology described in 9 CFR 113.52 is analogous to that for evaluation of bovine raw materials and includes provision for multiple subcultures, for Giemsa staining of fixed cells, for hemadsorption testing, and for use of specific immunostaining of fixed cells.
**CELL-BASED DETECTION OF MURINE MINUTE VIRUS**

Murine minute virus (MMV) is a mouse parvovirus that has been detected in biologics manufacturing involving Chinese hamster cell substrates. As with other paroviruses, MMV represents a special case in that the virus is difficult to inactivate using typical cleaning agents and is capable of surviving for prolonged periods of time on surfaces. Cell-based assays for MMV involve detector cell lines that are especially susceptible to this virus, such as 324K (a human cell) and A9 (a murine cell). Optimization for detection of a parvovirus also includes provision for detector cell subcultures to remain in log-phase division for a significant portion of the incubation period. Endpoints for detection of MMV include one or more of the following: cytopathic effect, hemagglutination of mouse and guinea pig erythrocytes, immunostaining, and polymerase chain reaction.

**CELL-BASED DETECTION OF INSECT-BORNE VIRUSES**

Insect-borne viruses include both viruses infectious only for insect cells (e.g., baculovirus) and those transmitted to mammalian cells via insect vectors (arboviruses). Detection of the former may be accomplished using an insect cell as a detector cell. Suitable substrates might include cells of *Spodoptera, Trichoplusia, Drosophila*, mosquito, or other insect origin. Such cells are typically cultured at lower temperatures (25° to 28°) relative to mammalian cells, and many of these cultures are suspension or semiadherent at best. Endpoints may include cytopathic effect, electron microscopy, and PCR.

Of more relevance to patient safety is the detection of arboviruses (insect-borne viruses infectious to animals and humans). This may be accomplished using a suitable mammalian detector cell. The Syrian hamster kidney cell (BHK-21) is a cell line that has shown susceptibility to a wide range of arboviruses. This cell line grows in a monolayer culture, and the endpoints that may be used include cytopathic effect, hemadsorption and hemagglutination, and PCR.

**CELL-BASED DETECTION OF HUMAN CYTOMEGALOVIRUS**

Human cytomegalovirus (CMV) is a slow-growing virus of special concern for biologics produced using human cell substrates. It may be detected in cell-based assays using human diploid detector cells such as WI-38 or MRC-5, provided that sufficiently long durations of incubation are employed (28 or more days). The endpoints include cytopathic effects and immunostaining and/or PCR.

**In Vivo Methods**

Intact and susceptible animals may serve as potential host organisms for detecting viruses in test samples. In this case, viral proliferation in the tissues of the host animal may be reflected as adverse health effects (including death) that can be monitored and recorded. Viral detection assays based on intact animals are intended to complement in vitro assays, because some viruses that do not cause a response in the in vitro assays may be detectable in the animal systems (and vice versa). Viral safety studies employing live animals must be performed in accordance with applicable regional guidelines for the ethical use of animals, using laboratories that are accredited for the housing of the animals.

**IN VIVO VIRAL SCREEN**

The in vivo viral screen is used primarily for cell bank, viral seed stock, and viral vaccine testing and is considered to complement the in vitro virus screening assay. Multiple animal species, as well as multiple injection routes, are employed to provide a broad range of host tissues and possible responses. A list of commonly used host animals, routes of inoculation, and target viruses are shown in Table 5.

<table>
<thead>
<tr>
<th>Host Animal</th>
<th>Route of Inoculation</th>
<th>Target Virus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suckling mouse</td>
<td>Intraperitoneal injection</td>
<td>Arboviruses</td>
</tr>
<tr>
<td></td>
<td>Intracranial injection</td>
<td>Coxsackie A and B</td>
</tr>
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<td></td>
<td><em>Per os</em> injection</td>
<td>Herpes simplex Type 1 and 2</td>
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<td></td>
<td></td>
<td>Togaviruses</td>
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<td></td>
<td></td>
<td>Junin</td>
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<tr>
<td></td>
<td></td>
<td>Herpes B</td>
</tr>
<tr>
<td>Adult mouse</td>
<td>Intraperitoneal injection</td>
<td>Rhabdoviruses</td>
</tr>
<tr>
<td></td>
<td>Intracranial injection</td>
<td>Togaviruses</td>
</tr>
<tr>
<td></td>
<td><em>Per os</em> injection</td>
<td>Lymphocytic choriomeningitis virus (LCMV)</td>
</tr>
</tbody>
</table>

Table 5. In Vivo Viral Screening Assays

Published on March 26, 2020
Table 5. In Vivo Viral Screening Assays (continued)

<table>
<thead>
<tr>
<th>Host Animal</th>
<th>Route of Inoculation</th>
<th>Target Virus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guinea pig</td>
<td>Intraperitoneal injection</td>
<td>Rhabdoviruses</td>
</tr>
<tr>
<td></td>
<td>Intracranial injection</td>
<td>LCMV</td>
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<td></td>
<td></td>
<td>Lassa</td>
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<td></td>
<td></td>
<td>Junin</td>
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<td></td>
<td></td>
<td>Marburg</td>
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<td></td>
<td></td>
<td>Ebola</td>
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<tr>
<td></td>
<td></td>
<td>Vaccinia viruses</td>
</tr>
<tr>
<td>Embryonated hens' eggs</td>
<td>Allantoic</td>
<td>Arboviruses</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Equine encephalomyelitis viruses</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chorio-allantoic membrane</td>
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<td></td>
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</tbody>
</table>

Following injection of the test sample, each animal model is monitored for an appropriate period of time that allows for the observation of clinical signs of viral infection. Any abnormality is investigated to determine the cause of the effect.

The suckling mice are observed for an appropriate period of time. Pooled homogenates from any surviving animals are then passaged into additional litters of suckling mice. The latter are observed for an additional period of time.

The guinea pigs are observed for clinical signs of viral infection and for injection site lesions. Necropsy for gross tubercular lesions is performed for certain types of test samples.

Allantoic fluids from eggs can be tested for hemagglutination of chicken, guinea pig, and human type-O erythrocytes. Additional fluids are pooled for each treatment group (test article and control), and these are passaged (inoculated) into a new group of embryonated eggs. Following an appropriate incubation period (typically measured in days), the allantoic fluids are again tested for hemagglutination of chicken, guinea pig, and human type-O erythrocytes. Following injection by the yolk sac route, the eggs are incubated for at least 9 days and are assessed for viability. The yolk sacs are then harvested and pooled for each group (test article and control), and a 10% solution of the resulting material is inoculated by the same route into a new group of embryonated eggs. The eggs are again incubated for an appropriate period of time (days) and are assessed for viability.

IN VIVO ASSAYS INTENDED TO DETECT SPECIFIC VIRUSES

Some in vivo assays are designed to detect, if not specific viruses, at least specific sets of viruses. The antibody production assays use the production of a humoral immune response in susceptible host animals inoculated with test samples. Viral antibody-free animals of the various species are injected with the test sample. At the end of an appropriate incubation period, one or more of a variety of endpoint assays may be performed to detect the generation of a humoral antibody response in the animal sera. Production in the animal of antibodies directed against a specific virus provides evidence of the presence of viral antigen or infectious virus in the test sample. This type of assay is typically used to ensure that rodent cell banks and viral seed stocks are free of adventitious viruses. Three antibody production assays, along with the route of injection and target viruses, are summarized in Table 6.
Table 6. In Vivo Antibody Production Assays

<table>
<thead>
<tr>
<th>Antibody Production Assay</th>
<th>Route of Injection</th>
<th>Target Virus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse antibody production (MAP) assay</td>
<td>Intranasal</td>
<td>Ectromelia</td>
</tr>
<tr>
<td></td>
<td>Intraperitoneal</td>
<td>Hantaan</td>
</tr>
<tr>
<td></td>
<td>Intracranial</td>
<td>Mouse K</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lactate dehydrogenase elevating virus</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lymphocytic choriomeningitis virus (LCMV)*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Murine minute virus</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mouse adenovirus</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mouse cytomegalovirus</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mouse encephalomyelitis virus type II</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mouse hepatitis virus</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Epizootic diarrhea of infant mice</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pneumonia virus of mice</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Polyomavirus</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reovirus type 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sendai</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mouse thymic virus</td>
</tr>
<tr>
<td>Hamster antibody production (HAP) assay</td>
<td>Intranasal</td>
<td>Lymphocytic choriomeningitis virus (LCMV)*</td>
</tr>
<tr>
<td></td>
<td>Intraperitoneal</td>
<td>Polyomavirus</td>
</tr>
<tr>
<td></td>
<td>Intracranial</td>
<td>Reovirus Type 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sendai</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Simian virus 5</td>
</tr>
<tr>
<td>Rat antibody production (RAP) assay</td>
<td>Intranasal</td>
<td>Hantaan</td>
</tr>
<tr>
<td></td>
<td>Intraperitoneal</td>
<td>Kilham rat virus</td>
</tr>
<tr>
<td></td>
<td>Intracranial</td>
<td>Mouse encephalomyelitis virus type II</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Polyomavirus</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reovirus type 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sendai</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Toolan’s H1 virus</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rat coronavirus/sialodacryoadenitis virus</td>
</tr>
</tbody>
</table>

*A group of test sample–injected mice is challenged with a known lethal dose of authentic LCMV. If this group of mice does not die from the challenge dose, a second group with twice the number of mice is used for a repeat challenge. If there are survivors in this group, the test sample is considered positive for LCMV.

Considerations for Validation, Matrix Qualification, and Quality Control of Cell- and Animal-Based Test Systems

Viral detection assays used to ensure the viral safety of human and animal therapeutics are expected to have undergone validation. The approach to the validation depends on the nature of the assay and associated regulatory compliance level.

Any assay should be sufficiently developed that it can be performed with an appropriate set of predetermined system suitability and acceptance criteria. These criteria usually include the use of relevant negative and positive controls but may also include requirements for linearity and meeting of a predetermined detection limit. The results constituting a positive or negative response in the assay should be established prior to execution of the validation. An assay used under GMP compliance is expected to have been validated according to appropriate guidelines.

An assay used to ensure the safety of a commercially marketed biological product must be further characterized for suitability in the presence of the specific product matrix. The matrix qualification study should address the potential for specific interference with the viral detection endpoints used in the assay, and typically involves spiking of one or more model viruses into the product matrix at levels approaching the limit of detection to ensure the absence of interference.

For quantitative detection assays, the detection limit should be probed. This usually involves spiking of the model virus(es) at decreasing amounts into medium or the product matrix. The lowest spiking level of the virus reliably detected is used as an approximation of the actual limit of detection of the assay. Experimental error for these cell culture-based assays is usually expected to be in the range of 0.5 to 1 log_{10}. The determination of a detection limit is less meaningful for limit tests and viral screening assays in general. For the latter, knowledge of the detection limit for one virus does not imply a similar limit for another.
virus. Since screening assays are not optimized for a specific virus, the limit of detection for the assay can vary greatly from one virus to another.

Animal-based viral detection systems are generally not subject to the requirements for validation, matrix qualification, use of positive controls, and determination of detection limit that regulatory agencies expect of cell-based and biochemical tests. The use of animals for safety testing is subject to the regional guidelines for the ethical use of animals, and the kinds of activities listed above generally are not considered appropriate use of animals. However, negative control animals are included in these assays, and retrospective validation or gap analysis based on historic incidence of system suitability failures or positive findings is sometimes possible.

**DETECTION OF VIRAL COMPONENTS**

Direct detection of viral components can provide a direct measurement of viral levels in a sample preparation. It has also become primarily important for detection or identification of viruses in biological products or in the raw materials used in their manufacture. Systems capable of identifying components unique to specific phases associated with viral latency and replication are now available. During interaction with their host cells, viruses may incorporate modified host molecules during the production of new intact virus particles, or they may induce discernable changes in host cell makeup or function.

Most immunological methods and reagents currently available detect the constituents of intact virions. It is the relative abundance of these proteins that makes them most amenable to the development of antibody-based reagents. Abundance also makes them optimal targets for detection of the virus. Recently developed targeting and detection reagents are aimed at minor viral components that may be found only during specific phases of replication. These allow a more detailed analysis of the stage of viral infection. The basic methodology for the detection of viral antigens is well established, but more recent innovations in materials and reagent development have broadened its application.

Developments in the targeting and detection of viral nucleic acid components have led to enzyme-based systems for the amplification of nucleic acids in vitro and in situ (see Nucleic Acid-Based Techniques—Amplification (1127)). The potential specificity of this detection method allows the examination of biological systems with a high degree of confidence for the presence or absence of a specific targeted virus. A wide variety of reagents, technology platforms, and methodologies are available. The aim of this section is to elaborate on the most common practices and platforms used in the detection of viral components.

**Sample Selection and Preparation for the Detection of Viral Components**

This subsection addresses general considerations for various types of test samples and the most common assay targets (viral proteins and nucleic acids). The target proteins may have varying levels of posttranslational modification (e.g., glycosylation, phosphorylation), and the target nucleic acids may be either RNA or DNA, single or double stranded. Therefore, it is important to have a basic understanding of the physicochemical nature of the virus under study so that the sample handling procedures support the detection of the target component. For detailed considerations regarding the extraction of nucleic acids, see the USP general information chapter Nucleic Acid-Based Techniques—Extraction, Detection, and Sequencing (1126).

**Types of Samples**

**CELLULAR**

When viral components are associated with intact cells, samples must be generated either as whole cell lysates or as subcellular fractions. Maintaining the temperature of the test samples at or near freezing (0° to 4°) during processing and limiting the time that samples are held in an unfrozen state will reduce the potential for loss of target antigens and nucleic acids due to cytosolic enzymes (proteases, nucleases) present in the cell lysates. Reagents that can inactivate or limit the activity of such enzymes may be used to prevent degradation of the target components, especially when exceptionally labile samples must be handled at room temperature. Centrifugation of intact cells allows for some additional manipulation of the sample matrix. The growth medium can be discarded and the cells suspended in a buffer formulated to enhance the recovery and detection of the targeted viral component. Collection and storage parameters should also account for the presence of cellular DNA. This can increase the viscosity of the sample, rendering it difficult to pipet.

**TISSUE CULTURE SUPERNATANT**

Depending on the stage of the infection and the type of virus involved, the conditioned medium may represent a preferred test sample. An advantage is that the presence of cellular debris can usually be reduced through use of a low-speed centrifugation (clarification) step. The main disadvantage is the potentially low concentration of the analyte, and therefore concentration of the sample may be required.

**PROCESS INTERMEDIATE (UNPROCESSED BULK HARVEST)**

In general, viral safety lot release testing is done at the bulk harvest stage prior to any purification. This is true regardless of whether the assay detects infectious virus or viral components. The presence of host cell DNA may need to be assessed in the case of biologics manufactured in animal cells.
Sample Stability and Matrix Effect

Sample stability is a key element in the successful detection of viral components. Protein structure can be altered by numerous environmental factors, including pH, ionic strength, solvents, detergents, temperature, and free radicals. In addition, complex biological matrices frequently contain proteolytic enzymes that can alter or destroy key antigenic features of a protein or peptide. Sample collection, storage, and handling must allow maintenance of the antigenic features targeted by reagent antibodies.

Conformational changes affecting the opportunity for antigen detection are difficult to address. Depending on the reagents required for detection, conformational changes may be required for antigen detection. For example, if antibodies are produced for an antigen detection system using native viral antigen, then unmasking and maintaining the conformation of the antigen throughout the sample preparation is essential. Conversely, if peptide fragments are used to produce antibody, then a denaturation step may be required to allow for effective antigen detection.

Conditions associated with sample preparation must be investigated in a combinatorial fashion whereby one parameter or component is varied while all others remain fixed. In this way, the formulation of lysis and processing buffers can be optimized for pH, ionic strength, and types of detergents and denaturants. The sample preparation steps must condition the targeted antigen in order to obtain the form most readily recognized by the reagent antibody.

The stability of nucleic acids in test samples is largely affected by nuclease activities present in the sample and the degree of protection provided by the intact structure of the virus particle. Encapsidated nucleic acids are particularly stable as long as the integrity of the capsid is maintained. Viral capsids are vulnerable to proteolytic digestion. Virus particles stored at ambient temperature as part of a complex biological matrix are especially susceptible to degradation by proteases. Storage at refrigerated temperatures (2° to 8°) for short periods of time or at temperatures below freezing can be used to limit proteolytic activity. When samples are stored at frozen temperatures, freeze-thaw cycles should be limited. Under conditions where an individual sample must be accessed multiple times, preparation of aliquots is advisable.

SAMPLE COLLECTION

In a biotechnology setting, sample collection is dictated by sampling plans that are established to meet regulatory requirements. Nucleic acid testing in association with an amplification step has the potential of detecting a virus at the earlier stages of infection. The use of nucleic acid amplification methods reduces the dependence on timing and the amount of material required, because the amplification process effectively boosts assay sensitivity by increasing the amount of target relative to background.

General aspects of nucleic acid sample preparation and stability are discussed in Nucleic Acid-Based Techniques—Extraction, Detection, and Sequencing (1126). The following section specifically addresses the unique aspects of viral sample selection and preparation.

SAMPLE STORAGE

Conditions for sample storage should be consistent with maintaining the antigenic properties of targeted viral proteins and/or preserving the nucleic acid content of the sample. The duration and temperature of storage is dictated also by cycle times associated with testing.

IMMUNE COMPLEX DISRUPTION

The masking of antigen epitopes may occur when other proteins associate at or near the epitope targeted by reagent antibodies. For viral antigens this may occur when the antigen comprises the structural component of the virus. Such viral antigens are likely to retain strong affinities for other viral proteins or the ability to exist in multimeric form under normal conditions for detection. Another obstacle for epitope recognition is the naturally occurring immune complex when reagent antibodies have been developed to detect native protein in a blood plasma matrix. Immune complexes consisting of viral antigens and host antibodies are normal in such physiologic samples. The stronger the host immune response, the more likely masking of antigen due to immune complex formation will occur. Methods aimed at the preparation of blood and plasma samples for detection should address the presence of preexisting immune complexes and incorporate steps designed to disrupt such complexes to improve the opportunity for viral antigen detection.

Detection of Viral Antigens

Viral capsid proteins are common targets for antigenic detection methods. Structural proteins that make up the framework of the viral core are often some of the most abundant viral proteins produced during viral replication. In nonenveloped viruses, the core structural proteins are likely to provide the dominant antigenic features. When the virus is enveloped, proteins associated with the envelope often provide key antigenic features. This section examines methods commonly used to detect viral antigens and addresses considerations aimed at optimizing formation of the appropriate immune complex.

ASSAYS USED FOR THE DETECTION OF VIRAL ANTIGENS

Immunologic methodologies used to detect viral antigens are based on the specificity and affinity of the antibody and viral antigen interaction. Of the various platforms available, one commonly employed in viral safety testing is immunofluorescent antibody staining. This lends some degree of viral specificity to the cell-based methods described in the first part of this chapter. Other techniques include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay, and Western blotting. The principles and general methods for these assays will be described in the USP general information chapters Immunological Test Methods—General Considerations (1102), Immunological Test Methods—Reagent Development (1103), Immunological Test Methods—Immunoassay Methodologies (1104), and Immunological Test Methods—Assay Design, Quality Control, and Data
Detection of Viral Nucleic Acids

The detection of viral nucleic acids provides another route for the determination of viral loads and for establishing the identity of a contaminant. Nucleic acids, like protein antigens, are essential components of viruses, and detectable quantities are usually indicative of viral presence. Detection assays can be designed and developed in some cases to parse viremia into phases, especially when the differentiation of nucleic acids along functional forms and configurations can provide clear insight into viral activity. Assays can be designed to determine whether viral DNA has been integrated into the host genome or still is encapsidated. Early viremia may be detected as viral mRNA transcripts prior to the accumulation of detectable viral particles. Nucleic acids may be the only detectable viral component of viruses that do not replicate well in tissue culture systems. Such systems may fail to produce mature virus particles, but the detection of viral transcripts can provide insight into whether the virus has the ability to infect the cell. Nucleic acid testing represents the most useful endpoint for the detection of certain viruses failing to cause responses using typical endpoints.

SAMPLE PREPARATION: SPECIAL CONSIDERATIONS FOR NUCLEIC ACID TESTING

The degradation of nucleic acids in samples can be limited through proper handling and storage practices and even enhanced by closely linking sample collection and preparation steps. In addition to preparing nucleic acids for further processing, denaturation is an important step toward stabilizing nucleic acids where storage temperatures extend above 0°C.

Denaturation and Dissociation of Virions (Viral Lysis)—Chaotropic detergents and salts can be important agents for disrupting and removing viral proteins that make up the viral capsid. Their addition can provide a useful first step when concentration of virions is not necessary or even possible. In sufficient quantity they rapidly denature the entire contents of a biologic sample, essentially fixing nucleic acid content through the inactivation of nucleases and other proteins that may affect sample stability. Saturated solutions containing guanidium salts, such as guanidine hydrochloride or guanidine isothiocyanate, are commonly used for the dissociation of viral nucleic acids from protein components. These solutions may be used alone or in combination with ionic detergents and other denaturants such as phenol. The main advantage of guanidinium salts is that they are readily removed during the concentration of viral nucleic acids using ethanol or isopropanol. Urea may also be used as a mild denaturing agent, although it does not perform as effectively as guanidine in its ability to disrupt virus particles.

Deproteinization—The removal of proteins during the processing of samples for the detection of viral nucleic acids is helpful in ensuring the reproducibility and robustness of assays, particularly those that rely on amplification to detect exceptionally low quantities of nucleic acids. Several strategies may be used to facilitate deproteinization of the sample; they are discussed in Nucleic Acid-Based Techniques—Extraction, Detection, and Sequencing (1126).
Recovery of Viral Nucleic Acids—Separation and recovery of extracted viral nucleic acid are important steps in the testing of nucleic acids. Nucleic acid yield and purity obtained at this step are critical determinants of assay robustness. Poor nucleic acid recovery and limited purification may inhibit amplification and detection reaction resulting in poor assay sensitivity. The development of high-yield, high-purity recovery steps is an important goal in the optimization of nucleic acid detection methods. Details on the general aspects of extraction and detection of nucleic acids are discussed in Nucleic Acid-Based Techniques—Extraction, Detection, and Sequencing (1126). However, if no inhibitory components are identified in the system, direct testing on an aliquot of a sample may increase the assay sensitivity.

Isolation of Viral DNA—Integrated viral genomes must be recovered and processed along with the genome of the host cell. The viral genome is analyzed within the context of the host genome, and therefore similarities between virus and host genomes must be accounted for to ensure that assay results are specific for the virus and do not simply reflect the presence of cross-reacting host genome sequences. Viral genomes that exist as episomal entities require similar consideration, but there may be opportunities during sample preparation to limit the amount of host nucleic acid present in the preparation.

Viral genomes exist as either DNA or RNA, or sometimes both: in the case of retroviruses the integrated genome is DNA, whereas the encapsidated form is RNA. The ability to differentiate among the various forms of viral nucleic acids can help to elucidate the course of specific viral infections. Assays for nucleic acid activity can differentiate readily between integrated and encapsidated genomes when the form of the viral nucleic acid varies between states, as in the case of retroviruses. Incorporation of specific nucleases into the assay methodology can be used to reduce or eliminate one form over the other. If viral genomes are known to integrate at specific sites within the host genome, primers and probes can be developed around the integration site and incorporate significant elements of both host and viral genomes. Some viral mRNAs contain splice sites, and the differentiation of spliced nucleic acid sequences from unspliced sequences creates a unique mechanism for determining the status of nucleic acid localization and infection.

Detection of Viral Genome Versus Viral Transcripts

Viral genomes exist as either DNA or RNA, or sometimes both: in the case of retroviruses the integrated genome is DNA, whereas the encapsidated form is RNA. The ability to differentiate among the various forms of viral nucleic acids can help to elucidate the course of specific viral infections. Assays for nucleic acid activity can differentiate readily between integrated and encapsidated genomes when the form of the viral nucleic acid varies between states, as in the case of retroviruses. Incorporation of specific nucleases into the assay methodology can be used to reduce or eliminate one form over the other. If viral genomes are known to integrate at specific sites within the host genome, primers and probes can be developed around the integration site and incorporate significant elements of both host and viral genomes. Some viral mRNAs contain splice sites, and the differentiation of spliced nucleic acid sequences from unspliced sequences creates a unique mechanism for determining the status of nucleic acid localization and infection.

CHARACTERIZATION OF DNA VIRAL GENOMES

Methods for the recovery and preparation of viral genomes for characterization depend on the state of the viral genome. If the genome has been incorporated into a cellular compartment, the recovery and preparation strategy must take into account the cellular components that make up the sample matrix. If the viral genome targeted for analysis is the encapsidated form, the methods must focus on recovery of the virus particle and must include additional steps aimed at extracting the nucleic acid from the individual particles. Identification and characterization of viral genomes require specific complementary nucleic acid probes and primers whose sequence will be dictated by available information about the sequence of the targeted viral nucleic acids and the type of assay that will be used. Determination of the sequence of the viral nucleic acid of interest usually provides the most unambiguous means for characterization. However, a number of methods can be used as simple indicators for the presence or absence of specific sequence-based characteristics. For example, melting curve profiles using short oligonucleotide sequences can be used to establish whether a specific viral genotype is present.

IDENTIFICATION AND GENOTYPE ANALYSIS

Nucleic acid testing is often used to identify viral isolates obtained from viral screening assays or to provide identity for viral stocks. Methods used for the identification of viral genomes are not unique to other applications in the field of molecular biology. Typically, an amplification step is required in order to achieve quantities for analysis. Amplified sequencing of the amplicons or application of a standard hybridization technique may be employed for more detail as to the nature of the amplified signal. For more details, refer to Nucleic Acid-Based Techniques—Amplification (1127).

HYBRIDIZATION TECHNIQUES

A variety of hybridization techniques are used to detect viral nucleic acid sequences, including Southern blot, Northern blot, DNAse/RNase protection, in situ hybridization, microarray technology, and other techniques. The description of these methods, which is well beyond the scope of this chapter, can be found in Nucleic Acid-Based Techniques—Extraction, Detection, and Sequencing (1126).
DETECTION OF ANTIBODIES TO VIRAL ANTIGENS

A variety of methods are available for detection and quantification of antibodies to viral agents, including neutralization, complement fixation, and immunoenzyme assays based on enzyme- or fluorescently labeled reagents. Although many details of the immunological methods mentioned above are beyond the scope of the chapter, this section addresses specific application aspects of viral antibody detection, including preparation and storage of test samples, common assay methods and platforms available, and specific examples of how these assays may be used to measure antibodies to specific viral agents.

Viral Structure Relative to Antigenic Composition and Selection of Antibody Assay

Mammalian viruses vary considerably in their nucleic acid content and thus the number of antigenic, virus-specific proteins produced.

Many viral proteins or glycoproteins are highly antigenic and induce a potent humoral immune response during natural infection, whether in humans or in animal models. In most cases, the immune system responds when the virus and its antigens appear in the extracellular fluid or on the infected cell membranes. The degree to which viral antigens are expressed is governed by the intracellular replication and protein synthesis of viruses in host organ tissues and by the several possible types of virus-host cell interaction. Antibodies produced as a result of natural viral infection are likely to represent the broadest response to antigens in their native state.

When selecting, developing, or evaluating an assay method for measurement of antibodies to viral proteins, the analyst must take into account the source of the antibodies and the method by which they were obtained or prepared. Details will be presented in Immunological Test Methods—Reagent Development (1103) and Immunological Test Methods—Immunoassay Methodologies (1104).

General Considerations Regarding Sample Preparation for Antibody Detection of Viral Antigens

Antibodies are relatively stable, but care must be taken to ensure the integrity of the test antibodies during sample preparation and storage. Serologic tests may be developed to measure antibodies to viral agents in unfractionated biological fluids. The possibility for matrix interference with the antibody detection method should be considered.

In general, biological test samples should be clarified by centrifugation or filtration, depending on their intended use. Serum samples should not be hemolyzed, lipemic, or icteric. In some cases the specimen should also be heat-treated to inactivate endogenous complement and other components.

Test samples should be processed as soon as possible. When it is necessary to store samples, most test samples should be stored at −20° for short-term storage and below −80° for long-term storage. For all samples, the stability of the material needs to be assessed experimentally. Aliquots of appropriate volume should be prepared in accordance with test procedures to avoid unnecessary freeze-thaw cycles.

Antibody Methods

This section discusses the primary methods for detecting antibodies directed against viral antigens. The assay methods often include a variety of alternative formats for the detection of antibody. Only the more commonly used formats for antibody detection are discussed in this section. Some methods, including fluorescent antibody assays and enzyme immunoassays, are widely applicable to the detection of antibodies to many different viral agents; others are limited to selected viruses having certain properties (e.g., hemagglutinins).

IMMUNOFLUORESCENCE MICROSCOPY FOR ANTIBODY DETECTION

When fluorescein isothiocyanate (FITC) is chemically coupled to an antibody molecule, the resulting FITC-labeled antibody can be used as a secondary antibody probe to detect the presence of a primary, virus-specific antibody bound to a virus-infected cell on a microscope slide (indirect immunofluorescence).

The indirect immunofluorescence or indirect fluorescent antibody (IFA) assay is one of the most basic and useful methods for detection of antibodies to viruses. The assay can be used to detect both virus-specific IgG- and IgM-class antibodies. When the assay is used to detect IgM antibodies, it usually requires the physical removal or inactivation/binding of IgG-class antibodies. In the absence of this step, the presence of IgM-specific antibody may be masked by excess IgG-specific antibody competing for primary binding sites on the substrate surface. IFA assays may be qualitative or quantitative.

The IFA for antibody to viral agents requires the use of virus-infected cells expressing viral antigens in cellular membranes. Viral stocks are prepared, titered, and used to infect permissive cells in tissue culture. The cells are harvested at appropriate times, washed, and spotted onto multiwell microscope slides at an appropriate density. Control slides are also prepared with noninfected cells. The slides are allowed to air-dry and then fixed in cold acetone. The fixed slides can then be stored under appropriate conditions for extended time periods. The stability of the viral antigens over time should be confirmed.

The test article to be examined for the presence of virus-specific antibodies can be applied to the slide, followed by an appropriate secondary antibody conjugated with a fluorescent tag that can be visualized under a fluorescent microscope. IgG- or IgM-specific antibodies can be distinguished by using the appropriately prepared secondary antibody.

Reading and correctly interpreting endpoints of IFA slides for antibody detection requires an experienced analyst, particularly when cellular location and fluorescent-staining patterns are critical for a specific virus. Such interpretation requires the use of appropriate controls and scoring or intensity of fluorescence. This is highly dependent on the quality of reagents, the consistency of the fluorescent microscopy and light source being used, and the experience of the analyst.
ENZYME IMMUNOASSAY FOR ANTIBODY DETECTION

The EIA and variations of it are the most widely used methods for the detection of viral antibodies in serum and other biological products. The most commonly used EIA for antibody detection is referred to as a noncompetitive solid phase EIA for antibody detection. The typical configuration of an EIA for antibody involves coating tubes or microwell plates with viral antigen(s), the addition of test serum or product to the tubes or wells, the binding of specific antibody in serum or product to antigen, and the detection of bound antibody by addition of a second antibody with binding affinity to the primary antibody, which is labeled to allow for its detection.

The assays can be specific to IgG- or IgM-class antibody or may detect total antibody. Assays for IgM may achieve improved specificity when performed as IgM-capture assays. These assays involve the use of plates or wells coated with anti-IgM antibody to capture total IgM in serum or product as a first step. Subsequently, viral antigen is added; it binds to the plate only if virus-specific IgM antibody has initially been captured, and it is detected by addition of a second labeled antibody specific to viral antigen. The assay is most often performed as a qualitative measure of the presence of an antibody for a specific virus. Sufficient replicates of both positive and negative control samples are required in order to determine the appropriate cut-off value and the assay acceptance criteria. A mean value of a test sample equal to or greater than the cut-off value is considered positive.

COMPLEMENT FIXATION TEST

Complement fixation has selective value in allowing for simultaneous assay of antibodies to a wide variety of viral agents. The procedure involves multiple variables consisting of two pairs of antigen–antibody reactions. The first reaction, between a known virus antigen and a specific antibody in the test sample, takes place in the presence of a predetermined amount of exogenous complement. The complement is removed by the antigen–antibody complex. The second antigen–antibody reaction consists of sheep red blood cells (SRBCs) and hemolysin (antibody against SRBC). When this indicator system is added to the reaction mixture, the sensitized SRBCs will lyse only in the presence of free complement. The extent of lysis of SRBCs is inversely correlated with the amount of the antibody in the test article.

The experimental procedure involves the optimal titration of concentrations of hemolytic serum, complement, and viral antigen, using chessboard format. If used as the test sample, human serum should be inactivated at 56° for 30 minutes to inactivate the endogenous complement activity. A number of important controls must be run along with the test, and results must be within limits before the test can be properly interpreted. These include the sensitivity of SRBCs to lysis and complement concentration used. The relative amount of virus-specific antibody present can be determined by testing serial dilutions of the serum or product. The complement-fixing titer is the reciprocal of the highest dilution that prevents 50% hemolysis.

NEUTRALIZATION FOR ANTIBODY DETECTION

Neutralization for the measurement of antibodies to viral agents is still one of the most valuable assays available because of its high specificity and its ability to detect neutralizing antibodies. Neutralization is defined as the loss of viral infectivity through the binding of specific antibodies to viral coat proteins (or envelope glycoproteins) on the surface of the infectious viral particle. The assay may be used to measure the presence of antibodies to a known virus in a serum or product sample, or conversely to identify an unknown virus by using a serum or product sample containing known antibodies.

Before performing a neutralization assay to measure the presence of antibodies in serum or product, a known virus must first be grown and titrated in the test system in which the neutralization assay will be performed. For viruses prepared in cell culture, this usually involves inoculating susceptible cell cultures with relatively low multiplicity of infection (MOI; <1 PFU per cell) and harvesting the infected cells when about 50% to 75% cytopathic effect (CPE) is demonstrated. The virus preparation is then titrated by preparing serial multifold dilutions and inoculating replicate tubes or plate cultures with a fixed volume of the virus preparation. The endpoint of the titration is the dilution of the virus that will infect 50% of the cell cultures inoculated. This endpoint is said to contain one 50% tissue culture infective dose (TCID₅₀) in the volume used. If the test system involves animal lethality, the endpoint is referred to as one 50% lethal dose (LD₅₀). The amount of virus used in the neutralization assay to follow is typically standardized to contain 100 TCID₅₀ or LD₅₀.

The test or host system used in neutralization assays is chosen on the basis of the specific virus to be tested and its ability to replicate in the system. The commonly used host systems include cell culture, embryonated chicken eggs, and mice. Cell culture is usually the preferred test system, because the viruses used in the neutralization assay usually readily replicate and produce CPE. Susceptible host cells are grown in monolayers in dishes or multiwell plate cultures. After the virus/neutralizing serum mixture is added, the cultures are overlaid with agar-containing medium to restrict spread of CPE and allow development of viral plaques. The prevention of plaque development is indicative of the presence of neutralizing antibody. Alternatively, neutralization can be performed in tube monolayer cultures or even in suspension tissue culture. Embryonated eggs may be used when the virus to be used or tested does not produce plaques in tissue culture systems. The route of inoculation and the endpoint depend on the virus.

Neutralization assays may be set up in various ways, depending on the specific virus of interest and the serum or product to be tested for neutralizing activity. In general, a fixed amount of infectious virus is preincubated with undiluted and serial dilutions of serum or product to be used for neutralizing activity and separately with preimmune serum or control product; this approach is referred to as the constant virus–varying serum method. Following preincubation, the mixtures are separately injected or added to the test system. Reduction in infectivity between test and control serum or product is scored in various ways, depending on the test system. The endpoint of the assay is generally defined as the highest dilution of the serum or product that neutralizes one-half of the initial viral inoculum, as calculated by Reed-Muench or the Spearman-Kärber method.

The titer of neutralizing antibody in the test serum or product is the reciprocal of the highest dilution that completely inhibits CPE or other virus effect in the test system. This dilution is said to contain 1 neutralizing antibody unit per unit volume used in the titration. When a serum or product known to contain neutralizing antibody is used in an assay to determine the identity of
an unknown virus, 20 neutralizing antibody units in a fixed volume are generally used in the assay. Positive and negative control sera must give expected reactivity in the assay.

HEMAGGLUTINATION INHIBITION (HAI)

A number of enveloped viruses, including the influenza and parainfluenza viruses, acquire protein receptors capable of binding RBCs (hemagglutinins) of various animal species on their surface as they bud through infected cell plasma membranes during viral maturation. In addition, some nonenveloped viruses such as adenoviruses and certain enteroviruses have hemagglutinin proteins in their outer capsid. This property allows for detection of a specific virus in a sample if a known specific antibody to the virus is available. Alternatively, the presence of antibody specific to the virus can be detected and quantitated by its ability to inhibit hemagglutination. This is the principle of the hemagglutination inhibition (HAI) test.

The HAI test for antibody is performed by making serial dilutions of the specimen to be tested and mixing the dilutions with a fixed amount of the virus or specific viral hemagglutinin protein in a tube or microtiter plate format. Indicator RBCs from the appropriate animal species are added, the suspension is mixed, and the tubes or plates are allowed to stand for a predetermined period. If specific antibody is present, the virus will bind and the RBCs will not agglutinate; they will settle to the bottom of the tube or plate and form an RBC “button”. If specific antibody is absent, the RBCs will be agglutinated by the virus and form a diffuse film. The titer of the serum or product is the reciprocal of the dilution that completely inhibits agglutination.

HAI is very useful for subtyping influenza virus isolates. A number of factors contribute to the potential variability of the HAI test. Certain serum samples and products may contain nonspecific inhibitors of RBC agglutinins, which may yield false-positive results. A number of procedures have been developed to remove such inhibitors, including adsorption and heat inactivation procedures. Specimens may also contain RBC agglutinins other than specific antibody, and these may contribute to false-negative results. Appropriate preparation and titration of reagents, including RBCs and viral hemagglutinin stocks and suspensions, is critical. In addition, controls for nonspecific agglutination or inhibitors of agglutination must be included in every assay.

WESTERN BLOT (OR IMMUNOBLOT) ASSAY FOR ANTIBODY DETECTION

The immunoblot, or Western blot, assay is a technique for the simultaneous detection of antibodies to various protein antigens of a given virus. The term recombinant immunoblot assay (RIBA) is applicable when the starting protein mixtures are recombinant proteins obtained from prokaryotic or eukaryotic expression systems instead of crude or partially purified virus from infected cells. The method is often used diagnostically as a supplementary or confirmatory test in situations where an initial assay for antibody lacks sufficient specificity or is known to be prone to false-positive results. This is especially important when the test is being used to diagnose an infection of clinical significance such as HIV or HCV infection.

A number of commercial immunoblot kits are available, particularly for viruses such as HIV and HCV; several have regulatory approval for diagnostic use. Alternatively, viral antigen preparations may be produced in-house or purchased, along with other reagents required for the assays. Careful control and/or sourcing of these reagents are critical to ensuring that compliance requirements are maintained.

For selected viral agents, there are generally accepted interpretive standards for the analysis of reactivity or positive results in an immunoblot assay. However, the presence of nonspecific bands may be due to antibody reactivity to cellular protein antigens caused by autoimmune diseases and/or the use of crude virus-infected cell proteins as antigen in the assay. Indeterminate reactions may also occur if only a limited number of specific antibody bands are observed.

Appropriate positive and negative control sera must be included in each assay and reactivity must be scored for both the presence and the intensity of expected protein bands.

APPLICATION OF THE ANTIBODY DETECTION METHODS TO SPECIFIC VIRUSES

Human blood-borne pathogens that may be present in infectious form in human donated blood used directly in the production of biological products are a concern because they may present a risk of transmission to others. Testing for virus-specific antibodies in donated blood serves as a screening procedure for the elimination of suspect units. Alternatively, the viruses may represent important agents for which human vaccines have been or are being developed. Thus the ability to detect virus-specific antibodies in an immunized individual or animal model may be important for demonstrating the efficacy of the vaccine. Currently, in the United States, a number of FDA-approved screening or definitive tests may be conducted on donated units of blood for evidence of the presence of agents of infectious diseases, including hepatitis B and C viruses, human immunodeficiency virus, and West Nile virus. In addition, plasma sent for fractionation before production of plasma-derived products is required to be tested for hepatitis A virus (HAV) and human parvovirus B-19.

GLOSSARY

Acceptance Criteria: Anticipated results, which may be numerical limits, ranges, or other characterization for the tests described. They establish the standards to which a drug substance or drug product should conform in order to be considered acceptable for its intended use.

Adventitious Agent: Acquired accidental contaminant in a cell line such as viruses and toxins; the agent is often infectious.

Amplicon: A segment of DNA generated by the PCR process whose sequence is defined by forward and reverse primers.

Antibody: An infection-fighting protein molecule that binds, neutralizes, and helps destroy foreign microorganisms or toxins. Also known as immunoglobulins, antibodies are produced by the immune system in response to antigens.

Antigen: Any agent that induces the production of an antibody and reacts specifically with it.

Assay Validation: A formal, archived demonstration of the analytical performance of an assay that provides justification for use of the assay for an intended purpose and a range of acceptable potency values.

Bioassay: Analytical method that uses living animals, cells, tissues, or organisms as test subjects.
Biologics: Products such as antitoxins, antivenins, blood, blood derivatives, immune serums, immunologic diagnostic aids, toxoids, vaccines, and related articles that are produced under license in accordance with the terms of the federal Public Health Service Act (58 Stat. 682) approved July 1, 1944, as amended, have long been known as “biologics.” However, in Table III, Part F, of the Act, the term “biological products” is applied to the group of licensed products as a whole. For Pharmacopeial purposes, the term “biologics” refers to those products that must be licensed under the Act and comply with Food and Drug Regulations—Code of Federal Regulations, Title 21 Parts 600–680, pertaining to federal control of these products (other than certain diagnostic aids), as administered by the Center for Biologics Evaluation and Research or, in the case of the relevant diagnostic aids, by the Center for Devices and Radiological Health of the federal Food and Drug Administration. [Definition from Biologics (1041), USP–NF vol. 30 (2007), p. 414.]

Biotechnology-Derived Product: Macromolecular article derived from biotechnology processes such as recombinant DNA (rDNA) technology, hybridoma technology, and the like.

Capsid: The outer protein shell of a virus particle.

Cell Bank: A defined population of cells, such as an immortalized cell line, grown by a defined process and cryopreserved in a defined process and within a defined passage number range. The assumption is that each vial from a cell bank is comparable and that when thawed and added to a manufacturing vessel (or an analytical assay), it will perform in a consistent way.

Chaotrope: A reagent that causes molecular structure to be disrupted; in particular, those formed by noncovalent forces such as hydrogen bonding, van der Waals interactions, and the hydrophobic effect.

Complement: A group of proteins in the blood that work in concert with other immune system proteins and cells (such as antibodies) in attacking foreign substances.

Complementary DNA: Complementary DNA. Two strands of nucleic acid that can hybridize by specific base pairing between the nucleotides.

Confluency: Refers to the point when 100% of the surface area of the vessel is covered in cells.

Cryopreservative: Reagent used to keep a cell alive in deep-frozen condition (usually in liquid nitrogen).

Cytopathic: Damaging to cells, causing them to exhibit signs of disease or cell death.

ELISA: Enzyme-linked immunosorbent assay. A biochemical technique used to detect the presence of an antibody or an antigen in a sample.

Endpoint Assay: An analytical method that measures the amount of accumulated product at the end of the assay.

Epitope: A molecular region on the surface of an antigen that is recognized by an antibody and can combine with the specific antibody produced by such a response; also called a determinant or an antigenic determinant.

Glycoprotein: Protein that contains sugar side chains added as a posttranslational process; the presence of sugar side chains often affects activity, antigenicity, and in vivo stability.

Host Cell Tropism: The range of susceptible cells that a particular microorganism can infect.

ICH: The International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use.

Limit of Detection (LOD): The lowest concentration of an analyte in a sample that can be detected, not quantitated. It is a limit test that specifies whether or not an analyte is above or below a certain value. It is expressed as a concentration at a specified signal-to-noise ratio, usually 2 or 3.

Mycoplasma: Parasitic microorganism that infects mammalian cells, possessing some characteristics of both bacteria and viruses. Prokaryotic microorganisms belong to the family Mycoplasmataceae, with no cell walls. They may grow attached or close to cell surfaces in the cytoplasm and subtly change the properties of the cells.

Passage: An operational procedure used to feed cultured cells, usually by providing fresh medium and dilution of cells in a new culture vessel. The number of such operations is referred to as the passage number. It is not the same as cell generation number, which is strictly related to cell doubling time.

qPCR: Quantitative polymerase chain reaction. A modification of the polymerase chain reaction used to measure the quantity of DNA, complementary DNA, or ribonucleic acid present in a sample. Like other forms of polymerase chain reaction, the process is used to amplify DNA samples via the enzyme DNA polymerase.

RT-PCR: Reverse transcriptase polymerase chain reaction. A variation of the PCR technique in which cDNA is made from RNA via reverse transcription. The cDNA is then amplified using standard PCR protocols.

Serotype: The kind of microorganism as characterized by testing for recognizable antigens on the surface of cells of the microorganism.

Spiking: Adding a known amount of analyte from a laboratory standard acting as a tracer to check a method for recovery or accuracy.

Syncytium: A multinucleated mass of cytoplasm that is not separated into individual cells.

System Suitability: The checking of a system to ensure system performance before or during the analysis of unknowns. Parameters such as plate count, tailing factors, resolution, and reproducibility are determined and compared against the specifications set for the method. These parameters are measured during the analysis of a system suitability sample, which is a mixture of main components and expected by-products.

TCID₅₀: 50% tissue culture infective dose. The level of dilution of a virus at which half of a series of laboratory wells contain active, growing virus.

Unprocessed Bulk Harvest: The pooled harvests of cell culture fluids that constitute a homogeneous mixture for manufacture into a unique lot of product.
APPENDIX

Relevant Regulatory References

3. 9 CFR 113.53.
4. 9 CFR 113.47.
5. 9 CFR 113.52.
6. 21 CFR 211.
7. 21 CFR 600.3.
8. Federal Food, Drug, and Cosmetic Act (FD&C Act), sections 201 (g) and (h).
11. Public Health Service Act, section 351 (i).
Reagents, Indicators and Solutions

Introduction

1. SCOPe

Reagents required in the tests and assay for U.S. Pharmacopeia and National Formulary articles and those required only in determining the quality of other reagents are listed in this section, with specifications appropriate to their intended uses.

As stated in General Notices, 6.70 Reagents, listing of reagents, indicators, and solutions in the U.S. Pharmacopeia in no way implies that they have therapeutic utility; thus, any reference to USP or NF in their labeling shall include also the term “reagent” or “reagent grade”.

Where a particular brand or source of a material, instrument, or piece of equipment, or the name and address of a manufacturer or distributor, is mentioned (ordinarily in a note or footnote), this identification is furnished solely for informational purposes as a matter of convenience, without implication of approval, endorsement, or certification.

1.1 ACS (American Chemical Society) Reagent Grade

Where it is directed to “Use ACS reagent grade”, it is intended that a grade meeting the corresponding specifications of the current edition of Reagent Chemicals, published by the American Chemical Society (ACS), shall be used.

1.2 Suitable Grade

In the cases where no ACS reagent monograph exists or if the reagent is available in different quality grades, each one specific for a particular application, it is directed to “Use a suitable grade”. The intent is that a suitable reagent grade available commercially shall be used.

Occasionally, additional test(s) augment the designation “suitable grade”, as indicated in the text. Listed also are some, but not all, reagents that are required only in determining the quality of other reagents. For those reagents that are not listed, satisfactory specifications are available in standard reference publications.

1.3 USP or NF or FCC Grade

In the instances in which a reagent required in a U.S. Pharmacopeia or National Formulary test or assay meets the requirements in the monograph for that article appearing in this U.S. Pharmacopeia or the National Formulary or the current edition of the Food Chemicals Codex (FCC), it suffices to refer to the corresponding monograph in one of the these three compendia. In such cases it is to be understood that the specifications are minimum requirements and that any substance meeting more rigid specifications for chemical purity is suitable.

2. PACKAGING AND STORAGE

Reagents and solutions should be preserved in tight containers made of resistant glass or other suitable material. Directions for storage in light-resistant containers should be carefully observed.

Stoppers and stopcocks brought into contact with substances capable of attacking or penetrating their surfaces may be given a protective coating of a thin film of a suitable lubricant unless specifically interdicted.

3. METAL-ION STANDARD SOLUTIONS

Atomic absorption and flame photometry require the use of a number of metal-ion standard solutions. While the individual monographs usually provide directions for preparation of these solutions, use of commercially prepared standardized solutions of the appropriate ions is permissible, provided that the analyst confirms the suitability of the solutions and has data to support their use.

4. DEFINITIONS

4.1 Reagents: Reagents are substances used either as such or as constituents of solutions.

4.2 Indicators: Indicators are reagents used to determine the specified endpoint in a chemical reaction, to measure hydrogen-ion concentration (pH), or to indicate that a desired change in pH has been effected. They are listed together with indicator test papers.

4.3 Buffer Solutions: Buffer solutions resist changes in the activity of an ion on the addition of substances that are expected to change the activity of that ion.

4.4 Colorimetric Solutions (CS): Colorimetric solutions are solutions used in the preparation of colorimetric standards for comparison purposes.

4.5 Test Solutions (TS): Test solutions are solutions of reagents in such solvents and of such definite concentrations as to be suitable for the specified purposes.

4.6 Volumetric Solutions (VS): Volumetric solutions are solutions of reagents of known concentration intended primarily for use in quantitative determinations.

5. CHROMATOGRAPHIC SOLVENTS AND CARRIER GASES

The chromatographic procedures set forth in the U.S. Pharmacopeia may require use of solvents and gases that have been especially purified for such use. The purpose may be (a) to exclude certain impurities that interfere with the proper conduct of the test procedure, or (b) to extend the life of a column by reducing the buildup of impurities on the column. Where solvents and gases are called for in chromatographic procedures, it is the responsibility of the analyst to ensure the suitability of the solvent or gas for the specific use. Solvents and gases suitable for specific high-pressure or other chromatographic uses are available as specialty products from various reagent supply houses, although there is no assurance that similar products from different suppliers are of equivalent suitability in any given procedure.
REAGENT SPECIFICATIONS

Acetic Acid, Glacial, CH₃COOH—60.05 [64-19-7]—Use ACS reagent grade.

Acetone (Propanone; Dimethylformaldehyde), CH₃COCH₃—58.08 [67-64-1]—Use ACS reagent grade.
[Note—For UV spectrophotometric determinations, use ACS reagent grade. Acetone suitable for use in UV Spectrophotometry.]

Acetonitrile (Methyl Cyanide; Cyanomethane), CH₃CN—41.05 [75-05-8]—Use ACS reagent grade.

Adamantane, C₁₀H₁₆—136.23 [281-23-2]
Melting Range (741): between 270° and 271°.

Alcohol, Dehydrated (Absolute Alcohol), C₂H₅OH—46.07—Use ACS reagent grade Ethyl Alcohol, Absolute.

Ammonia Water, 25 Percent [1336-21-6]—Use a suitable grade.

Ammonium Acetate, NH₄C₂H₃O₂—77.08 [631-61-8]—Use ACS reagent grade.

Butyl Alcohol (1-Butanol; Normal Butyl Alcohol), CH₃(CH₂)₂CH₂OH—74.12 [71-36-3]—Use ACS reagent grade.

Citric Acid, C₆H₈O₇·H₂O—210.14 [5949-29-1]—Use ACS reagent grade.

Formic Acid, HCOOH—46.03 [64-18-6]—Use ACS reagent grade Formic Acid, 88 Percent.

n-Heptane, Chromatographic, C₇H₁₆—100.21 [142-82-5]
—Clear, colorless, volatile, flammable liquid consisting essentially of C₇H₁₆. Practically insoluble in water; soluble in absolute alcohol. Miscible with ether, with chloroform, with benzene, and with most fixed and volatile oils. Use a suitable grade, chromatographic or HPLC, with a content of not less than 99%.

n-Hexane, C₆H₁₄—86.18 [110-54-3]—for use in spectrophotometry—Use Hexanes.

Hexane, Solvent (Petroleum Benz; Petroleum Ether, Ligroin) [8032-32-4]—Clear, volatile liquid. Practically insoluble in water; soluble in absolute alcohol. Miscible with ether, with chloroform, with benzene, and with most fixed and volatile oils. [Caution—It is dangerously flammable. Keep it away from flames, and store in tight containers in a cool place.]
Use ACS reagent grade Petroleum Ether.

Hydrochloric Acid, HCl—36.46 [7647-01-0]—Use ACS reagent grade.

Isopropyl Alcohol (2-Propanol), (CH₃)₂CHOH—60.10 [67-63-0]—Use ACS reagent grade.
[Note—For use in assays and tests involving UV spectrophotometry, use ACS reagent grade Isopropyl Alcohol suitable for use in UV Spectrophotometry.]

Methanol (Methyl Alcohol), CH₃OH—32.04 [67-56-1]—Use ACS reagent grade.

Methylene Chloride (Dichloromethane), CH₂Cl₂—84.93 [75-09-2]—Use ACS reagent grade Dichloromethane.

Naphthalene, C₁₀H₈—128.17 [91-20-3]—Monoclinic prismatic plates, or white scales or powder. Use a suitable grade with a content of not less than 98%.

Phenol [108-95-2]—Use ACS reagent grade.

Phosphoric Acid, H₃PO₄—98.00 [7664-38-2]—Use ACS reagent grade.

Polyoxyethylene 10 Lauryl Ether (Decaethylene Glycol Monododecyl Ether) C₁₂H₂₅O₁₀—628.86 [9002-92-0]—Use a suitable grade.
[Note—A suitable grade is available as catalog number P9769 at http://www.sigma-aldrich.com.]

Polysorbate 80 (Polyoxyethylene Sorbitan Monooleate; Tween 80; Polyoxyethylene Sorbitan Monolaurate) —micellar average molecular weight 79,000 [9005-63-6]—Use a suitable grade.

Potassium Bromide, KBr—119.00 [7758-02-3]—Use ACS reagent grade.

Potassium Chloride, KCl—74.55 [7447-40-7]—Use ACS reagent grade.

Potassium Hydroxide, KOH—56.11 [1310-58-3]—Use ACS reagent grade.

Potassium Iodide, KI—166.00 [7681-11-0]—Use ACS reagent grade.

Potassium Phosphate, Dibasic ( Dipotassium Hydrogen Phosphate; Dipotassium Phosphate), K₂HPO₄—174.18 [7758-11-4]—Use ACS reagent grade.

Potassium Phosphate, Monobasic (Potassium Biphosphate; Potassium Dihydrogen Phosphate), KH₂PO₄—136.09 [7778-77-0]—Use ACS reagent grade.
[Note—Certified Potassium Dihydrogen Phosphate is available from the National Institute of Standards and Technology, Washington, DC, www.nist.gov, as standard sample No. 186.]

Sodium Bicarbonate, NaHCO₃—84.01 [144-55-8]—Use ACS reagent grade.

Sodium Chloride, NaCl—58.44 [7647-14-5]—Use ACS reagent grade.

Sodium Dodecyl Sulfate (Sodium Lauryl Sulfate), C₁₂H₂₅SO₄Na—288.38 [151-21-3]—Light yellow, crystalline powder.

Sodium Hydroxide, NaOH—40.00 [1310-73-2]—Use ACS reagent grade.

Sodium Nitrite, NaNO₂—69.00 [7632-00-0]—Use ACS reagent grade.

Sodium Phosphate, Monobasic (Sodium Biphosphate; Sodium Dihydrogen Phosphate; Acid Sodium Phosphate; Monosodium Orthophosphate), NaH₂PO₄·H₂O—137.99 [10049-21-5]—Use ACS reagent grade.
**Solutions**

**Buffer Solutions**

1. **DEFINITION**
   A solution is said to be buffered if it resists changes in the activity of an ion on the addition of substances that are expected to change the activity of that ion. Buffers are substances or combinations of substances that impart this resistance to a solution. Buffered solutions are systems in which the ion is in equilibrium with substances capable of removing or releasing the ion.

2. **BUFFER CAPACITY**
   It refers to the amount of material that may be added to a solution without causing a significant change in ion activity. It is defined as the ratio of acid or base added (in gram-equivalents/L) to the change in pH units. The capacity of a buffered solution is adjusted to the conditions of use, usually by adjustment of the concentrations of buffer substances.

3. **USES**
   Buffers are used to establish and maintain an ion activity within narrow limits. The most common systems are used for the following:
   1. To establish hydrogen-ion activity for the standardization of pH meters
   2. In the preparation of dosage forms
   3. In analytical procedures
   4. To maintain stability of various dosage forms

   Buffers used in physiological systems are carefully chosen so as not to interfere with pharmacological activity of the medicament or normal function of the organism.

4. **STANDARD BUFFER SOLUTIONS**
   Standard solutions of definite pH are readily available in buffer solutions prepared from the appropriate reagents. Buffer solutions, buffer tablets, and buffer solids may be obtained from commercial sources in convenient prepackaged form.

   **4.1 PREPARATION**
   Previously dry the crystalline reagents at 110°–120° for 1 h, except for boric acid and sodium acetate trihydrate.

   Where water is specified for solution or dilution of test substances in pH determinations, use carbon dioxide-free water.

   Store the prepared solutions in chemically resistant, tight containers such as Type 1 glass bottles. Use the solutions within 3 months.

   Standard buffer solutions for various ranges between pH 1.2 and 10.0 may be prepared by appropriate combinations of the solutions described herein, used in the proportions shown in the table below.

   The volumes shown in the table below are for 200 mL of buffer solution, except for Acetate Buffer where the volumes are for 1000 mL of buffer solution and for Citrate Buffer where the volumes are for 100 mL of buffer solution.

   1. **Hydrochloric Acid, 0.2 M** and Sodium Hydroxide, 0.2 M: Prepare and standardize as directed in Volumetric Solutions.
   2. **Potassium Biphthalate, 0.2 M**: 40.85 g/L of potassium biphthalate in water
   3. **Potassium Phosphate, Monobasic 0.2 M**: 27.22 g/L of monobasic potassium phosphate in water
   4. **Boric Acid and Potassium Chloride, 0.2 M**: 12.37 g/L of boric acid and 14.91 g/L of potassium chloride in water
   5. **Potassium Chloride, 0.2 M**: 14.91 g/L of potassium chloride in water

---

**Sodium Phosphate, Tribasic, Na₂PO₃·12H₂O—380.12 [10101-89-0]**—Use ACS reagent grade.

**Sodium Sulfate, Anhydrous, Na₂SO₄—142.04 [7757-82-6]**—Use ACS reagent grade.

For use in assaying alkaloids by gas–liquid chromatography, it conforms to the following additional test.

**Suitability for Alkaloid Assays**: Transfer about 10 mg of atropine, accurately weighed, to a 25-mL volumetric flask, dissolve in and dilute with alcohol to volume. Pipet 3 mL of the solution into each of two 60-mL separators, and add to each 10 mL of water, 1 mL of 1 N sodium hydroxide, and 10 mL of chloroform. Shake thoroughly, and allow the layers to separate. Filter the organic phase from one separator, through phase-separating paper, previously washed with 5 mL of chloroform, supported in a funnel, and collect the filtrate in a suitable container. Add 10 mL of chloroform to the separator, shake thoroughly, and filter the organic layer through the same phase-separating paper, collecting and combining the filtrates in the same container. Designate the combined filtrates as Solution A. Filter the organic phase from the second separator through 30 g of the Anhydrous Sodium Sulfate, supported on a pledget of glass wool in a small funnel, and previously washed with chloroform, and collect the filtrate in a suitable container. Add 10 mL of chloroform to the separator, shake thoroughly, and filter the organic layer through the same portion of anhydrous sodium sulfate, collecting and combining the two filtrates in the same container. Designate the combined filtrates as Solution B. Evaporate the two solutions in vacuum to a volume of about 1 mL. Inject an accurately measured volume of Solution A into a suitable gas chromatograph, and record the peak height. Repeat the determination with a second accurately measured volume of Solution A, record the peak height, and obtain the average of the two results. In a similar manner, determine the peak height of two portions of Solution B, and obtain the average of the results. The average value obtained for Solution B is within 5.0% of the value obtained for Solution A.

Under typical conditions, the gas chromatograph contains a 4-mm × 1.2-m glass column packed with 3% phase G3 on packing S1A. After curing and conditioning, the measured volume of Solution A is introduced to the column temperature at 210°, the injector port temperature at 225°, and the detector block temperature at 240° during the determinations. The carrier gas is helium, flowing at a rate of 60 mL per minute.

**Sulfuric Acid, H₂SO₄—98.08 [7664-93-9]**—Use ACS reagent grade.

**Tetrahydrofuran, Stabilizer-Free**: Use a suitable grade.


**Toluene (Toluol), C₇H₈CH₃—92.14 [108-88-3]**—Use ACS reagent grade.

**Water**—Use USP Purified Water.
6. **Acetic Acid, 2 N:** Prepare and standardize as directed in *Volumetric Solutions*.

7. **Citric Acid, 0.1 M:** 21.01 g/L of citric acid in water

8. **Sodium Citrate, 0.1 M:** 29.41 g/L of sodium citrate dihydrate in water

Buffers—See *Buffer Solutions* under *Solutions*.

### Test Solutions

**Ammonia TS**—It contains between 9.5% and 10.5% of NH₃. Prepare by diluting 350 mL of Ammonia Water, Stronger (see in the section, *Reagents*) with water to make 1000 mL.

**Ammonia Solution, Diluted**—Use Ammonia TS.

**5 N Sodium Hydroxide TS**—Transfer 20 g of sodium hydroxide to a 100-mL volumetric flask. Dissolve in about 80 mL of water. Cool and dilute with water to volume.

### Volumetric Solutions

**0.1 N Hydrochloric Acid VS, HCl,** —36.46

3.646 g in 1000 mL

Dilute 8.5 mL of hydrochloric acid with water to 1000 mL.

Standardization: Accurately weigh about 0.5 g of tromethamine, dried according to the label instructions or, if this information is not available, dried at 105° for 3 h. Dissolve in 50 mL of water, and add 2 drops of bromocresol green TS. Titrate with 0.1 N hydrochloric acid to a pale yellow endpoint. Each 12.114 mg of tromethamine is equivalent to 1 mL of 0.1 N hydrochloric acid.

\[
N = \frac{\text{mg tromethamine}}{121.14 \times \text{mL HCl}}
\]

[NOTE—If this volumetric solution is used in a qualitative application such as pH adjustment, dissolution medium, or diluent, its standardization is not required.]

**1 N Sodium Hydroxide VS NaOH,** —40.00

40.00 g in 1000 mL

Dissolve 162 g of sodium hydroxide in 150 mL of carbon dioxide-free water, cool the solution to room temperature, and filter through hardened filter paper. Transfer 54.5 mL of the clear filtrate to a tight, polyolefin container, and dilute with carbon dioxide-free water to 1000 mL.

Standardization: Accurately weigh about 5 g of potassium biphthalate, previously crushed lightly and dried at 120° for 2 h, and dissolve in 75 mL of carbon dioxide-free water. Add 2 drops of phenolphthalein TS, and titrate with the sodium hydroxide solution to the production of a permanent pink color. Each 204.22 mg of potassium biphthalate is equivalent to 1 mL of 1 N sodium hydroxide.

\[
N = \frac{\text{g KHC₆H₄O₄}}{0.20422 \times \text{mL NaOH solution}}
\]

[NOTE—(1) Solutions of alkali hydroxides absorb carbon dioxide when exposed to air. They should be preserved in bottles having well-fitted, suitable stoppers, provided with a tube filled with a mixture of sodium hydroxide and lime (soda-lime tubes) so that air entering the container must pass through this tube, which will absorb the carbon dioxide. (2) Prepare solutions of lower concentration (e.g., 0.1 N, 0.01 N) by quantitatively diluting accurately measured volumes of the 1 N solution with sufficient carbon dioxide-free water to yield the desired concentration.]

Restandardize the solution frequently.

[NOTE—If this volumetric solution is used in a qualitative application such as pH adjustment, dissolution medium, or diluent, its standardization is not required.]

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### CHROMATOGRAPHIC REAGENTS

#### Packings

**L1**—Octadecyl silane chemically bonded to porous or nonporous silica or ceramic microparticles, 1.5 to 10 µm in diameter, or a monolithic silica rod.

**L7**—Octylsilane chemically bonded to totally porous or superficially porous silica particles, 1.5–10 µm in diameter, or a monolithic silica rod.

**L11**—Phenyl groups chemically bonded to porous silica particles, 1.5–10 µm in diameter, or a monolithic silica rod.

**L26**—Butyl silane chemically bonded to totally porous or superficially porous silica particles, 1.5–10 µm in diameter.

#### Phases

**G1**—Dimethylpolysiloxane oil.

**G16**—Polyethylene glycol compound (av. mol. wt. about 15,000). A high molecular weight compound of polyethylene glycol with a diepoxide linker. [NOTE—Available commercially as Polyethylene Glycol Compound 20M, or as Carbowax 20M, from suppliers of chromatographic reagents.]

**G27**—5% Phenyl-95% methylpolysiloxane.
**Composition of Standard Buffer Solutions**

### Hydrochloric Acid Buffer
Place 50 mL of the potassium chloride solution in a 200-mL volumetric flask, add the specified volume of the hydrochloric acid solution, then add water to volume.

<table>
<thead>
<tr>
<th>pH</th>
<th>1.2</th>
<th>1.3</th>
<th>1.4</th>
<th>1.5</th>
<th>1.6</th>
<th>1.7</th>
<th>1.8</th>
<th>1.9</th>
<th>2.0</th>
<th>2.1</th>
<th>2.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2 M HCl, mL</td>
<td>85.0</td>
<td>67.2</td>
<td>53.2</td>
<td>41.4</td>
<td>32.4</td>
<td>26.0</td>
<td>20.4</td>
<td>16.2</td>
<td>13.0</td>
<td>10.2</td>
<td>7.8</td>
</tr>
</tbody>
</table>

### Acid Phthalate Buffer
Place 50 mL of the potassium biphthalate solution in a 200-mL volumetric flask, add the specified volume of the hydrochloric acid solution, then add water to volume.

<table>
<thead>
<tr>
<th>pH</th>
<th>2.2</th>
<th>2.4</th>
<th>2.6</th>
<th>2.8</th>
<th>3.0</th>
<th>3.2</th>
<th>3.4</th>
<th>3.6</th>
<th>3.8</th>
<th>4.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2 M HCl, mL</td>
<td>49.5</td>
<td>42.2</td>
<td>35.4</td>
<td>28.9</td>
<td>22.3</td>
<td>15.7</td>
<td>10.4</td>
<td>6.3</td>
<td>2.9</td>
<td>0.1</td>
</tr>
</tbody>
</table>

### Neutralized Phthalate Buffer
Place 50 mL of the potassium biphthalate solution in a 200-mL volumetric flask, add the specified volume of the sodium hydroxide solution, then add water to volume.

<table>
<thead>
<tr>
<th>pH</th>
<th>4.2</th>
<th>4.4</th>
<th>4.6</th>
<th>4.8</th>
<th>5.0</th>
<th>5.2</th>
<th>5.4</th>
<th>5.6</th>
<th>5.8</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2 M NaOH, mL</td>
<td>3.0</td>
<td>6.6</td>
<td>11.1</td>
<td>16.5</td>
<td>22.6</td>
<td>28.8</td>
<td>34.1</td>
<td>38.8</td>
<td>42.3</td>
</tr>
</tbody>
</table>

### Phosphate Buffer
Place 50 mL of the monobasic potassium phosphate solution in a 200-mL volumetric flask, add the specified volume of the sodium hydroxide solution, then add water to volume.

<table>
<thead>
<tr>
<th>pH</th>
<th>5.8</th>
<th>6.0</th>
<th>6.2</th>
<th>6.4</th>
<th>6.6</th>
<th>6.8</th>
<th>7.0</th>
<th>7.2</th>
<th>7.4</th>
<th>7.6</th>
<th>7.8</th>
<th>8.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2 M NaOH, mL</td>
<td>3.6</td>
<td>5.6</td>
<td>8.1</td>
<td>11.6</td>
<td>16.4</td>
<td>22.4</td>
<td>29.1</td>
<td>34.7</td>
<td>39.1</td>
<td>42.4</td>
<td>44.5</td>
<td>46.1</td>
</tr>
</tbody>
</table>

### Alkaline Borate Buffer
Place 50 mL of the boric acid and potassium chloride solution in a 200-mL volumetric flask, add the specified volume of the sodium hydroxide solution, then add water to volume.

<table>
<thead>
<tr>
<th>pH</th>
<th>8.0</th>
<th>8.2</th>
<th>8.4</th>
<th>8.6</th>
<th>8.8</th>
<th>9.0</th>
<th>9.2</th>
<th>9.4</th>
<th>9.6</th>
<th>9.8</th>
<th>10.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2 M NaOH, mL</td>
<td>3.9</td>
<td>6.0</td>
<td>8.6</td>
<td>11.8</td>
<td>15.8</td>
<td>20.8</td>
<td>26.4</td>
<td>32.1</td>
<td>36.9</td>
<td>40.6</td>
<td>43.7</td>
</tr>
</tbody>
</table>

### Acetate Buffer
Place the specified amount of sodium acetate ($\text{NaC}_2\text{H}_3\text{O}_2 \cdot 3\text{H}_2\text{O}$) in a 1000-mL volumetric flask, add the specified volume of the acetic acid solution, then add water to volume, and mix.

<table>
<thead>
<tr>
<th>pH</th>
<th>4.1</th>
<th>4.3</th>
<th>4.5</th>
<th>4.7</th>
<th>4.9</th>
<th>5.1</th>
<th>5.2</th>
<th>5.3</th>
<th>5.4</th>
<th>5.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH (measured)</td>
<td>4.10</td>
<td>4.29</td>
<td>4.51</td>
<td>4.70</td>
<td>4.90</td>
<td>5.11</td>
<td>5.18</td>
<td>5.30</td>
<td>5.40</td>
<td>5.48</td>
</tr>
<tr>
<td>$\text{NaC}_2\text{H}_3\text{O}_2 \cdot 3\text{H}_2\text{O}$, g</td>
<td>1.5</td>
<td>1.99</td>
<td>2.99</td>
<td>3.59</td>
<td>4.34</td>
<td>5.08</td>
<td>5.23</td>
<td>5.61</td>
<td>5.76</td>
<td>5.98</td>
</tr>
<tr>
<td>2 N $\text{CH}_3\text{COOH}$, mL</td>
<td>19.5</td>
<td>17.7</td>
<td>14.0</td>
<td>11.8</td>
<td>9.1</td>
<td>6.3</td>
<td>5.8</td>
<td>4.4</td>
<td>3.8</td>
<td>3.0</td>
</tr>
</tbody>
</table>

Published on March 26, 2020
Citrate Buffer

Mix 0.1 M Citric Acid with 0.1 M Sodium Citrate in the proportions given below.

<table>
<thead>
<tr>
<th>pH</th>
<th>3.0</th>
<th>3.2</th>
<th>3.4</th>
<th>3.6</th>
<th>3.8</th>
<th>4.0</th>
<th>4.2</th>
<th>4.4</th>
<th>4.6</th>
<th>4.8</th>
<th>5.0</th>
<th>5.2</th>
<th>5.4</th>
<th>5.6</th>
<th>5.8</th>
<th>6.0</th>
<th>6.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 M Citric Acid, mL</td>
<td>82.0</td>
<td>77.5</td>
<td>73.0</td>
<td>68.5</td>
<td>63.5</td>
<td>59.0</td>
<td>54.0</td>
<td>49.5</td>
<td>44.5</td>
<td>40.0</td>
<td>35.0</td>
<td>30.5</td>
<td>25.5</td>
<td>21.0</td>
<td>16.0</td>
<td>11.5</td>
<td>8.0</td>
</tr>
<tr>
<td>0.1 M Sodium Citrate, mL</td>
<td>18.0</td>
<td>22.5</td>
<td>27.0</td>
<td>31.5</td>
<td>36.5</td>
<td>41.0</td>
<td>46.0</td>
<td>50.5</td>
<td>55.5</td>
<td>60.0</td>
<td>65.0</td>
<td>69.5</td>
<td>74.5</td>
<td>79.0</td>
<td>84.0</td>
<td>88.5</td>
<td>92.0</td>
</tr>
</tbody>
</table>
General Provisions and Requirements Applying to Specifications, Tests, and Assays of the Food Chemicals Codex

The General Provisions provide, in summary form, guidelines for the interpretation and application of the standards, tests and assays, and other specifications of the Food Chemicals Codex and make it unnecessary to repeat throughout the book those requirements that are pertinent in numerous instances. Where exceptions to the General Provisions are made, the wording in the individual monograph or general test chapter takes precedence and specifically indicates the directions or the intent.

TITLE OF BOOK

The title of this book, including its supplements, is the Food Chemicals Codex, Twelfth Edition. It may be abbreviated to FCC 12. Where the term FCC is used without further qualification in the text of this book, it applies to the Food Chemicals Codex, Twelfth Edition.

APPROPRIATE USE OF THE FOOD CHEMICALS CODEX

As a compendium that addresses known food ingredients used in food products either in the United States or internationally, the FCC has many practical applications in industry, research, and academia. The FCC does not, however, provide information on the regulatory status or safety of food chemicals, nor does the presence or absence of standards for a particular food ingredient indicate in any way USP’s endorsement (or lack thereof) of that item for use in foods or food processing. It is the responsibility of the user to determine the safety and regulatory status of a particular food ingredient for any specific application.

FCC standards have been developed in cooperation with regulatory authorities and industry in the United States and elsewhere both under the stewardship of the Institute of Medicine and, more recently, USP. While USP makes great efforts to dialog with the U.S. Food and Drug Administration (FDA) regarding creating or revising monograph standards in the FCC, USP has no official legislative authority to establish legal requirements for food ingredients in the United States. The FCC serves as a resource for companies that manufacture, process, purchase, or use food ingredients and seek to determine appropriate minimum standards for components of their food products.

The structure and format of the FCC monographs and informational chapters allow users to quickly access the following types of information:

• General information about food ingredients
• Chemical information specific to food ingredients
• Information regarding laboratory method validation components
• Guidance for establishing and using Good Manufacturing Practices
• Validated testing methods (including enzyme assays and methods that use highly-characterized reference standards)
• Minimum standards for identity, purity, and quality of food ingredients

Food ingredient manufacturers, processors, and purchasers often use the FCC’s standards as the basis for establishing minimum requirements for identity, purity, and quality of their ingredients. FCC standards are also used to define these parameters within commercial purchase agreements between buyers and sellers of ingredients and food and, thus, help to promote food quality and food safety programs in industry. The validated test methods included in the FCC can be used to demonstrate the identity, quality, and purity of food ingredients, or they can be a starting point in developing new test methods. Manufacturers, processors, and purchasers of food ingredients will find these validated test methods useful, as will regulatory agency labs, contract labs, and students of chemistry or food science. In addition to being a resource for purchasing and quality control operations, portions of the FCC are useful to quality assurance groups and can serve as references for internal Standard Operating Procedures (SOPs) and quality manuals used by the food industry. The FCC is an excellent resource that may be used to provide important information in order

1 For further information about the legal status of FCC, see Legal Recognition of FCC Standards, in the Preface.
to ascertain identity, quality, and purity of ingredients. In addition, the FCC can be an important part of a food manufacturer or purchaser's comprehensive food quality program and it provides a common basis for evaluations of food ingredients in all aspects of food research and the food industry.

FCC SPECIFICATIONS

FCC specifications are presented in monograph form for each substance or group of related substances. They are designed to ensure that food ingredients have the specified identity and a sufficiently high level of quality to be safe under usual conditions of intended use in foods or in food processing. Thus, FCC specifications generally represent acceptable levels of quality and purity of food-grade ingredients available in the United States (or in other countries or instances in which FCC specifications are recognized).

Manufacturers, vendors, and users of FCC substances are expected to exercise good manufacturing practices (GMPs) (see General Information). They are also expected to establish food safety assurance systems such as Hazard Analysis and Critical Control Points (HACCP) to ensure that FCC substances are safe and otherwise suitable for their intended use. FCC substances must meet applicable regulatory requirements, including microbiological criteria, for safety and quality.

The name of the substance on a container label, plus the designation “Food Chemicals Codex Grade,” “FCC Grade,” or simply “FCC,” is a representation by the manufacturer, vendor, or user of the substance that at the time of shipment, the substance conforms to the specifications in the effective edition of FCC, including any Supplement that is effective at the time. When an FCC substance is available commercially in solution form as a component of a mixture and there is no provision in the FCC for such solution or mixture, the manufacturer, vendor, or user may indicate on the label that the product contains substances meeting FCC specifications by use of the initials “FCC” after the name of those components that meet the FCC specifications. For the labeling of FCC substances in which added substances are permitted, see Added Substances.

Added Substances FCC specifications are intended for application to individual substances and not to proprietary blends or other mixtures. Some specifications, however, allow “added substances” (i.e., functional secondary ingredients such as anti-caking agents, antioxidants, diluents, emulsifiers, and preservatives) intentionally added when necessary to ensure the integrity, stability, utility, or functionality of the primary substance in commercial use.

If an FCC monograph allows such additions, each added substance must meet the following requirements: (1) it is permitted for use in foods by the FDA or by the responsible government agency in other countries; (2) it is of appropriate food-grade quality and meets the requirements of the FCC, if listed therein; (3) it is used in an amount not to exceed the minimum required to impart its intended technical effect or function in the primary substance; (4) its use will not result in concentrations of contaminants exceeding permitted levels in any food as a consequence of the affected FCC primary substance’s being used in food; and (5) it does not interfere with the tests and assays prescribed for determining compliance with the FCC requirements for the primary substance, unless the monograph for the primary substance has provided for such interferences. Where added substances are specifically permitted in an FCC substance, the label shall state the name(s) of any added substance(s).

Adding substances not specifically provided for and mentioned by name or function in the monograph of an FCC substance will cause the substance to no longer be designated as an FCC substance. Such a combination is a mixture to be described by disclosure of its ingredients, including any that are not FCC substances.

Title of Monograph The titles of FCC monographs are in most instances the common or usual names. FCC specifications apply equally to substances bearing the main titles, synonyms listed under the main titles, and names derived by transposition of definitive words in main titles. The nomenclature used for flavoring agents may not be consistent with other authoritative sources.

Molecular Structures and Chemical Formulas Molecular structures, chemical formulas, and formula weights immediately following titles are included for the purpose of information and are not to be considered an indication of the purity of the substance. Molecular formulas given in specifications, tests, and assays, however, denote the pure chemical entity.

CAS Number If available, Chemical Abstracts Service (CAS) registry numbers are included for informational purposes. Additional CAS numbers may be relevant.

INS Numbers If available, numbers assigned by the Codex Alimentarius Commission under the International Numbering System for Food Additives are included for informational purposes.

FEMA Numbers If available, numbers assigned by the Flavor and Extract Manufacturers Association of the United States (FEMA) are included for informational purposes.

UNII The Unique Ingredient Identifier (UNII) is a nonproprietary, free, unique, unambiguous, nonsemantic, alphanumeric identifier based on a substance’s molecular structure and/or descriptive information issued through the joint FDA/USP Substance Registration System (SRS) to support health information technology initiatives for substances in drugs, biologics, foods, and devices.

Alternative Analytical Procedures Although the tests and assays described constitute procedures upon which the specifications of the FCC depend, analysts are not prevented from applying alternative procedures if supporting data shows that the procedures used will produce results of equal or greater accuracy. In the event of the doubt or disagreement concerning a substance purported to comply with the specifications of the FCC, only the methods described herein are applicable and authoritative.

Labeling For purpose of compliance with FCC monographs, "labeling" means all labels and other written, printed, or graphic matter (1) on any article of any of its containers or wrappers or (2) accompanying such article, or otherwise provided by vendors to purchasers for purposes of product identification.

Sulfiting agents If an FCC substance contains 10 mg/kg or more of any sulfiting agent, the presence of such sulfiting agent shall be indicated on the labeling.

Requirements for Listing Substances in the FCC

The FCC is intended to be an international compendium of food ingredient standards. The requirements for listing substances in the FCC are as follows: (1) the substance is permitted for use in food or in food processing in the United States or in other countries, (2) it is commercially available, and (3) suitable specifications and analytical test procedures are available to determine its identity and purity.
GENERAL SPECIFICATIONS AND STATEMENTS

Certain specifications and statements in the monographs of the FCC are not amenable to precise description and accurate determination within narrow limiting ranges. Because of the subjective or general nature of these specifications, good judgment, based on experience, must be used in interpreting and attaching significance to them.

Description Characteristics described and statements made in the Description section of a monograph are not requirements, but are provided as information that may assist with the overall evaluation of a food ingredient. The section includes a description of physical characteristics such as color and form and information on stability under certain conditions of exposure to air and light. It may also include odor terms that are general descriptors and do not necessarily indicate the source of the material. Statements in this section may also cover approximate indications of properties such as solubility (see below) in various solvents, pH, melting point, and boiling point, with numerical values modified by “about,” “approximately,” “usually,” “-,” and other comparable nonspecific terms.

Function A statement of function is provided to indicate the technical effect(s) of the substance in foods or in food processing or a principle application such as “Nutrient”. The statement is not intended to limit in any way the choice or use of the substance or to indicate that it has no other utility. The term “Source of...” is used to describe the function of materials that may, following ingestion, exhibit a functional effect on the human body, in a manner similar to that of some nutrients. These substances are products of an emerging science, and a comprehensive understanding of their beneficial effects has yet to be developed. The inclusion of monographs for these materials should not be interpreted as implying an endorsement of the claimed potential health or other benefits.

Odorless This term, when used in describing a flavoring material, applies to the examination, after exposure to air for 15 min, of about 25 g of the material that has been transferred from the original container to an open evaporating dish of about 100-mL capacity. If the package contains 25 g or less, the entire contents should be examined.

Packaging and Storage Statements in monographs relating to packaging and storage are advisory in character and are intended only as general information to emphasize instances where deterioration may be accelerated under adverse packaging and storage conditions, such as exposure to air, light, or temperature extremes, or where safety hazards are involved. Additionally, to reduce the risk of intentional or accidental introduction of undesirable materials into food substances, containers should be equipped with tamper-resistant closures.

Cool Place A cool place is one where the temperature is between 8° and 15° (46° and 59°F). Alternatively, it may be a refrigerator, unless otherwise specified in the monograph.

Excessive Heat Any temperature above 40° (104°F).

Storage under Nonspecific Conditions Where no specific storage directions or limitations are provided in the individual monograph, the conditions of storage and distribution include protection from moisture, freezing, and excessive heat. Containers should be stored in secure areas when not in use to reduce the possibility of tampering.

Containers The container is the device that holds the substance and that is or may be in direct contact with it. The immediate container is in direct contact with the substance at all times. The closure is a part of the container. Closures should be tamper-resistant and tamper-evident. The container should not interact physically or chemically with the material that it holds so as to alter its strength, quality, or purity. The food ingredient contact surface of the container should comply with relevant regulations promulgated under the Federal Food, Drug, and Cosmetic Act (or with applicable laws and regulations in other countries). Polynsaturated fats and oils are particularly susceptible to oxidation when stored in metal containers, at elevated temperatures, and/or in open containers. Oxidation can be minimized by storing them in closed, nonmetal containers with minimal headspace or flushed with nitrogen gas.

Light-Resistant Container A light-resistant container is designed to prevent deterioration of the contents beyond the prescribed limits of strength, quality, or purity under the ordinary or customary conditions of handling, shipments, storage, and sale. A colorless container may be made light resistant by enclosing it in an opaque carton or wrapper (see also Apparatus, below).

Well-Closed Container A well-closed container protects the contents from extraneous solids and from loss of the chemical under the ordinary or customary conditions of handling, shipment, storage, and sale.

Tight Container A tight container protects the contents from contamination of extraneous liquids, solids, or vapors; from loss of the chemical; and from efflorescence, deliquescence, or evaporation under the ordinary or customary conditions of handling, shipment, storage, and sale, and is capable of tight reclosure.

Product Security Tamper-evident packaging closures and security tags should be used. Containers that appear to have been opened or otherwise altered by unauthorized persons should not be used until the purity of the substance has been confirmed.

Solubility Statements included in a monograph under a heading such as Solubility in Alcohol express exact requirements and constitute quality specifications. Statements relating to solubility given in the Description, however, are intended as information regarding approximate solubilities only and are not to be considered as exact FCC-quality specifications. Such statements are considered to be of minor significance as a means of identification or determination of purity. For those purposes, dependence must be placed upon other FCC specifications.

Approximate solubilities given in the Description are indicated by the following descriptive terms:

<table>
<thead>
<tr>
<th>Descriptive Term</th>
<th>Parts of Solvent Required for 1 part of Solute</th>
</tr>
</thead>
<tbody>
<tr>
<td>Very Soluble</td>
<td>less than 1</td>
</tr>
<tr>
<td>Freely Soluble</td>
<td>from 1 to 10</td>
</tr>
<tr>
<td>Soluble</td>
<td>from 10 to 30</td>
</tr>
<tr>
<td>Sparingly Soluble</td>
<td>from 30 to 100</td>
</tr>
<tr>
<td>Slightly Soluble</td>
<td>from 100 to 1000</td>
</tr>
<tr>
<td>Very Slightly Soluble</td>
<td>from 1000 to 10,000</td>
</tr>
<tr>
<td>Practically Insoluble or Insoluble</td>
<td>more than 10,000</td>
</tr>
</tbody>
</table>

Soluble substances, when brought into solution, may show slight physical impurities, such as fragments of filter paper, fibers, and dust particles unless excluded by definite tests or other requirements. Significant amounts of black specks, metallic chips, glass fragments, or other insoluble matter are not permitted.
TESTS AND ASSAYS

Every substance in commerce that claims or purports to conform to FCC, when tested in accordance with its tests and assays, meets all of the requirements in the FCC monograph defining it.

The methods and analytical procedures described in the FCC are designed for use by properly trained personnel in a suitably equipped laboratory. In common with many laboratory procedures, test methods in the FCC frequently involve hazardous materials. In performing the test procedures and assays in the FCC, safe laboratory practices must be followed. This includes the use of precautionary measures, protective equipment, and work practices consistent with the chemicals and procedures used. Before undertaking any assay or procedures described in the FCC, the individual should be aware of the hazards associated with the chemicals and of the procedures and means of protecting against them. Material Safety Data Sheets, which contain precautionary information related to safety and health concerns, are available from manufacturers and distributors of chemicals such as USP and should provide helpful information about the safe use of such chemicals. Certain chemical reagents specified in FCC test procedures may be considered to be hazardous or toxic by the Occupational Safety and Health Administration, by the Environmental Protection Agency (under provisions of the Toxic Substances Control Act), or by health authorities in other countries. Where such reagents are specified, the analyst is encouraged to investigate the use of suitable substitute reagents, as appropriate, and to inform the USP FCC Liaison (fcc@usp.org) of the results so obtained.

Analytical Samples In the description of tests and assays, the approximate quantity of the analytical sample to be used is usually indicated. The quantity actually used, however, should not deviate by more than 10% from the stated amount. Tests or assays sometimes call for a sample taken to be “previously dried.” Where a test for Loss on Drying or Loss on Ignition is included in a monograph, the conditions specified for these procedures are to be used to dry the sample prior to performing the test procedure or assay, unless otherwise specified. Often, the results of tests or assays that do not call for use of a “previously dried” sample are expressed as calculated on the dried, anhydrous, or ignited basis. In such cases, a test for Loss on Drying, Water, or Loss on Ignition is included in the monograph and the result of such a test is used for the calculation on the dried, anhydrous, or ignited basis, provided that any moisture or other volatile matter in the undried sample does not interfere with the specified test procedures and assays. In editions of the FCC prior to the Seventh edition, the terms “exactly,” “accurately weighed,” and “accurately measured” are used in connection with gravimetric or volumetric measurements and linked directly to a sample weight or volume. These terms indicate that an operation should be carried out within the limits of error prescribed under Volumetric Apparatus or Weights and Balances, Appendix I. In the Seventh edition and each subsequent edition, these terms have been removed from most monographs, to be more concise. Nonetheless, it shall be understood that all quantitative measurements are to be performed “accurately” and in conformance with the provisions in Volumetric Apparatus or Weights and Balances, Appendix I, unless otherwise indicated by qualifiers such as “about” or by the particular nature of the test procedure. The word “transfer,” when used in describing tests and assays, means that the procedure should be carried out quantitatively.

Apparatus With the exception of volumetric flasks and other exact measuring or weighing devices, directions to use a definite size or type of container or other laboratory apparatus are intended only as recommendations, unless otherwise specified. Where an instrument for physical measurement, such as a thermometer, spectrophotometer, or gas chromatograph, is designated by its distinctive name or trade name in a test or assay, a similar instrument of equivalent or greater sensitivity of accuracy may be employed. An instrument may be substituted for the specified instrument if the substitute uses the same fundamental principles of operation and is of equivalent or greater sensitivity and accuracy. These characteristics must be validated as appropriate. Where low-actinic or light-resistant containers are specified, clear glass containers that have been rendered opaque by application of a suitable coating or wrapping may be used. Where a particular brand or source of a material, instrument, or piece of equipment, or the name and address of the manufacturer, or distributor, is mentioned (ordinarily in a footnote), this identification is furnished solely for informational purposes as a matter of convenience, without implication of approval, endorsement, or certification.

Atomic Weights The atomic weights used in computing formula weights and volumetric and gravimetric factors stated in tests and assays are those recommended in 1991 by the IUPAC Commission on Isotopic Abundances and Atomic Weights.

Blank Tests Where a blank determination is specified in a test or assay, it is to be conducted using the same quantities of the same reagents and by the same procedure repeated in every detail except that the substance being tested is omitted. A residual blank titration may be stipulated in tests and assays involving a back titration in which a volume of a volumetric solution larger than is required to react with the sample is added, and the excess of this solution is then titrated with a second volumetric solution. Where a residual blank titration is specified or where the procedure involves such a titration, a blank is run as directed in the preceding paragraph. The volume of the titrant consumed in the back titration is then subtracted from the volume required for the blank. The difference between the two, equivalent to the actual volume consumed by the sample, is the corrected volume of the volumetric solution to be used in calculating the quantity of the substance being determined.

Centrifuge Where the use of a centrifuge is indicated, unless otherwise specified, the directions are predicated on the use of the apparatus having an effective radius of about 20 cm (8 in) and driven at a speed sufficient to clarify the supernatant layer within 15 min. If necessary, determine the gravity by using the equation \( g = \left(\frac{(rpm \times 2 \times \pi \times r)}{60}\right) \times \frac{t_m}{980} \), in which rpm is the rotor speed and \( t_m \) is the mean radius, in cm, of the tube holding the sample in the rotor.

Desiccators and Desiccants The expression “in a desiccator” means using a tightly closed container of appropriate design in which a low moisture content can be maintained by means of a suitable desiccant. Preferred desiccants include anhydrous calcium sulfate, magnesium perchlorate, phosphorus pentoxide, and silica gel.

Filtration Where it is directed to “filter,” without further qualification, the intent is that the liquid be filtered through suitable filter paper or an equivalent device until the filtrate is clear.

Identification The tests described under this heading in monographs are designed for application to substances
taken from labeled containers and are provided only as an aid to substantiate identification. These tests, regardless of their specificity, are not necessarily sufficient to establish proof of identity, but failure of a substance taken from a labeled container to meet the requirements of a prescribed identification test means that it does not conform to the requirements of the monograph.

**Indicators** The quantity of an indicator solution used should be 0.2 mL (approximately 3 drops) unless otherwise directed in a test or assay.

**mg/kg and Percent** The term "mg/kg" is used in expressing the concentration of trace amounts of substances, such as impurities, up to 10 mg/kg. Above 10 mg/kg, percent (by weight) is used. For example, a monograph requirement equivalent to 20 mg/kg is expressed as 0.002%, or 0.0020%, depending on the number of significant figures justified by the test specified for use in conjunction with the requirement.

**Microbial Limit Tests** The FCC directly references the procedures in the FDA Bacteriological Analytical Manual (BAM) (http://www.fda.gov/Food/default.htm) for its microbial limit tests. Where the sample size is not defined in the limit, the results are based on the sampling procedures described in BAM.

**Negligible** The term "negligible," as used in some Residue on Ignition specifications, indicates a quantity not exceeding 0.5 mg.

**Pressure Measurements** The term "mm Hg" used with respect to pressure within an apparatus, or atmospheric pressure, refers to the use of a suitable manometer or barometer calibrated in terms of the pressure exerted by a column of mercury of the stated height.

**Reagents** Specifications for reagents are not included in the FCC. Unless otherwise specified, reagents required in tests and assays should conform to the specifications of the current editions of Reagent Chemicals: American Chemical Society Specifications or in the section on Reagent Specifications in the United States Pharmacopeia. Reagents not covered by any of these specifications should be of a grade suitable to the proper performance of the method of test or assay involved.

**Acids and Ammonium Hydroxide** When ammonium hydroxide, glacial acetic acid, hydrochloric acid, hydrofluoric acid, nitric acid, phosphoric acid, or sulfuric acid is called for in tests and assays, reagents of ACS grade and strengths are to be used. (These reagents sometimes are called "concentrated," but this term is not used in the FCC.)

**Alcohol, Ethyl Alcohol, Ethanol** When one of these substances is called for in tests and assays, use ACS-grade Ethyl Alcohol (95%) or USP-grade Alcohol.

**Alcohol Absolute, Anhydrous Alcohol, Dehydrated Alcohol** When one of these substances is called for in tests and assays, use ACS-grade Ethyl Alcohol, Absolute or USP-grade Dehydrated Alcohol.

**Water** When water is called for in tests and assays or in the preparation of solutions, it shall have been prepared by distillation, ion-exchange treatment, or reverse osmosis. When water, Carbon Dioxide-Free When this type of water is called for, it shall have been boiled vigorously for 5 min or more, and allowed to cool while protected from absorption of carbon dioxide from the atmosphere. "Deoxygenated" or "degassed water" is water that has been treated to reduce the content of dissolved air by suitable means, such as by boiling vigorously for 5 min and cooling while protected from air or by the application of ultrasonic vibration.

**Reference Standards** Test and assay results are determined on the basis of comparison of the test sample with a reference standard that has been freed from or corrected for volatile residues or water content, as instructed on the reference standard label. The requirements for any new FCC standards, tests, or assays for which a new USP or FCC Reference Standard or Authentic Substance is specified are not in effect until the specified Reference Standard or Authentic Substance is available. If a reference standard is required to be dried before use, transfer a sufficient amount to a clean, dry vessel. Do not use the original container as the drying vessel, and do not dry a reference standard repeatedly at temperatures above 25°. Where the titrimetric determination of water is required at the time a reference standard is to be used, proceed as directed in the Karl Fisher Titrimetric Method under Water Determination, Appendix IIB. Unless a reference standard bears a specific potency or content, assume that the reference standard is 100% pure in the compendial application. [Directions for use printed on the label text of USP and FCC reference standards are lot-specific, and they take precedence over any other indication listed in the FCC.]

**Significant Figures** When tolerance limits are expressed numerically, the values are significant to the number of digits indicated. Record the observed or calculated analytical result with only one digit included in the decimal place to the right of the last place in the limit expression. If this digit is smaller than 5, eliminate it and increase the preceding digit unchanged. If this digit is greater than 5, eliminate it and increase the preceding digit by one. For example, a requirement of not less than 96.0% would not be met by a result of 95.94%, but would be met by results of 95.96% or 95.95%, both of which would be rounded to 96.0%. When a range is stated, the upper and lower limits are inclusive so that the range consists of the two values themselves, properly rounded, and all values between them.

**Solutions** Prepare all solutions, unless otherwise specified, with water prepared by distillation, ion-exchange treatment, reverse osmosis, or as otherwise indicated in the monograph. Expressions such as "1:10" or "10%" mean that 1 part by volume of a liquid or 1 part by weight of a solid is to be dissolved in a volume of the diluent or solvent sufficient to make the finished solution 10 parts by volume. Directions for the preparation of colorimetric solutions (CS), test solutions (TS), and volumetric solutions (VS), are provided in the section on Solutions and Indicators. Prepare a volumetric solution to have a normality (molarity) within 10% of the stated value and to be standardized to four significant figures. When volumetric equivalence factors are provided in tests and assays, the term "0.X N(M)" is understood to mean a VS having a normality (molarity) of exactly 0.X000 N(M). If the normality (molarity) of the VS employed in a particular procedure differs from 0.X000, apply an appropriate correction factor.

**Specific Gravity** Numerical values for specific gravity, unless otherwise noted, refer to the ratio of the weight of a substance in air at 25° to that of an equal volume of water at the same temperature. Determine specific gravity by any reliable method, unless otherwise specified.

**Temperatures** Unless otherwise specified, temperatures are expressed in Celsius (centigrade) degrees, and all measurements are to be made at 25°, unless otherwise directed.

**Time Limits** Unless otherwise specified, allow 5 min for a reaction to take place when conducting limit tests for trace
impurities such as chloride or iron. Expressions such as "exactly 5 min" mean that the stated period should be accurately timed.

**Tolerances** Minimum purity tolerance limits presented in monographs neither bar the use of lots of articles that more nearly approach 100% purity nor constitute a basis for a claim that such lots exceed the quality prescribed by the FCC. When no monograph assay tolerance is given, the assay should show the equivalent of not more than 100.5%.

**Trace Impurities** Tests for inherent trace impurities are provided to limit such substances to levels that are consistent with good manufacturing practice and that are safe and otherwise unobjectionable under conditions in which the food additive or ingredient is customarily employed. It is impossible for FCC to provide limits and tests in each monograph for the detection of all possible unusual or unexpected impurities, the presence of which would be inconsistent with good manufacturing practice. The limits and tests provided in FCC are those considered to be necessary according to currently recognized methods of manufacture and are based on information available to or provided to the Food Ingredients Expert Committee. If other methods of manufacture or other than the usual raw materials are used, or if other possible impurities may be present, additional tests may be required and should be applied, as necessary, by the manufacturer, vendor, or user to demonstrate that the substance is suitable for its intended application. Such tests should be submitted to the USP FCC Liaison (fcc@usp.org) for consideration for inclusion in the FCC.

**Vacuum** The unqualified use of the term "in vacuum" means a pressure at least as low as that obtainable by an efficient aspirating water pump (not higher than 20 mm Hg).

**Water and Loss on Drying** In general, for compounds containing water of crystallization or adsorbed water, a limit test, to be determined by the Karl Fischer Titrimetric Method, is provided under the heading Water. For compounds in which the Loss on Drying may not necessarily be attributable to water, a limit test, to be determined by other methods, is provided under the heading Loss on Drying.

**Weighing Practices**

**Constant Weight** A direction that a substance is to be "dried to constant weight" means that the drying should continue until two consecutive weighings differ by not more than 0.5 mg/g of the sample taken, the second weighing to follow an additional hour of drying. The direction "ignite to constant weight" means that the ignition should be continued at 800 ± 25°, unless otherwise specified, until two consecutive weighings do not differ by more than 0.5 mg/g of the sample taken, the second weighing to follow an additional 15 min of ignition.

**Tared Container** When a tared container, such as a glass filtering crucible, a porcelain crucible, or a platinum dish, is called for in an analytical procedure, it shall be treated as is specified in the procedure, e.g., dried or ignited for a specified time or to constant weight, cooled in a desiccator as necessary, and weighed accurately.

**Weights and Measures, Symbols and Abbreviations:** The International System of Units (SI), to the extent possible, is used in most specifications, tests, and assays in this edition of FCC. The SI metric units, and other units and abbreviations commonly employed, are as follows:

- ° = degrees Celsius
- kg = kilogram
- g = gram
- mg = milligram
- µg = microgram
- ng = nanogram
- pg = picogram
- L = liter
- mL = milliliter
- µL = microliter
- m = meter
- cm = centimeter
- dm = decimeter
- mm = millimeter
- µm = micrometer (0.001 mm)
- nm = nanometer
- ~ = approximately
- C = coulomb
- A = ampere
- V = volt
- mV = millivolt
- W = watt
- dc = direct current
- ft = foot
- in = inch
- in³ = cubic inch
- gal = gallon
- lb = pound
- oz = ounce
- mEq = milliequivalents
- mg/kg = parts per million (by weight)
- µg/kg = parts per billion (by weight)
- ng/kg = parts per trillion (by weight)
- psi = pounds per square inch
- psia = pounds per square inch absolute
- kPa = kilopascal
- sp. gr. = specific gravity
- b.p. = boiling point
- m.p. = melting point
- id = inside diameter
- od = outside diameter
- h = hour
- min = minute
- s = second
- N = normality
- M = molarity
- mM = millimolar
- mmol = millimole
- µM = micromolar
- µmol = micromole
- CFU = colony-forming unit(s)
- ACS = American Chemical Society
- AOAC = AOAC International
- AOCS = American Oil Chemists Society
- ASTM = ASTM (American Society for Testing and Materials) International
- CAS = Chemical Abstracts Service
- CFR = Code of Federal Regulations (U.S.)
- FDA = United States Food and Drug Administration
- FEMA = Flavor and Extract Manufacturers Association of the United States
INS = International Numbering System of the Codex Alimentarius
IUPAC = International Union of Pure and Applied Chemistry
NIST = National Institute of Standards and Technology
UNII = Unique Ingredient Identifier (as defined by US FDA)
FCC Monographs

Ethyl Alcohol

First Published: Prior to FCC 6
Alcohol
Ethanol

C₂H₆O  Formula wt 46.07
CAS: [64-17-5]

**DESCRIPTION**
Ethyl Alcohol occurs as a clear, colorless, mobile liquid. It is miscible with water, with ether, and with chloroform. It boils at about 78° and is flammable. Its refractive index at 20° is about 1.364.

**ASSAY**
- **SPECIAL GRAVITY:** Determine by any reliable method (see General Provisions).
  
  **Acceptance criteria:** NMT 0.8096 at 25°/25° (equivalent to 0.8161 at 15.56°/15.56°), and equivalent to NLT 94.9% by volume (92.3% by weight) of C₂H₆O

**IMPURITIES**

Inorganic Impurities
- **LEAD, Lead Limit Test, Atomic Absorption Spectrophotometric Graphite Furnace Method, Method I, Appendix IIIB**
  
  **Sample:** 10 g
  
  **Acceptance criteria:** NMT 0.5 mg/kg

Organic Impurities
- **FUSIL OIL**
  
  **Sample:** 10 mL
  
  **Analysis:** Mix the Sample with 1 mL of glycerin and 1 mL of water, and allow to evaporate from a piece of clean, odorless, absorbent paper.
  
  **Acceptance criteria:** No foreign odor is perceptible when the last traces of alcohol leave the paper.

- **KETONES, ISOPROPYL ALCOHOL**
  
  **Sample:** 1 mL
  
  **Analysis:** Transfer the Sample, 3 mL of water, and 10 mL of mercuric sulfate TS to a test tube; mix; and heat in a boiling water bath.
  
  **Acceptance criteria:** No precipitate forms within 3 min.

- **METHANOL**
  
  **Sample:** 10 mL
  
  **Analysis:** Transfer 10 mL of sulfuric acid into a small Erlenmeyer flask, cool to 10° and, with constant agitation, add the Sample, dropwise.
  
  **Acceptance criteria:** The mixture is colorless or has no more color than either the acid or the sample before mixing.

- **SUBSTANCES REDUCING PERMANGANATE**
  
  **Sample:** 20 mL
  
  **Analysis:** Transfer the Sample, previously cooled to 15°, to a glass-stoppered cylinder, add 0.1 mL of 0.1 N potassium permanganate, mix, and allow to stand for 5 min.
  
  **Acceptance criteria:** The pink color does not entirely disappear.

**SPECIFIC TESTS**
- **ACIDITY (AS ACETIC ACID)**
  
  **Sample:** 25 mL
  
  **Analysis:** Add 2 drops of methyl red TS to 25 mL of water, add 0.5 mL of phenolphthalein TS, and then add 0.02 N sodium hydroxide to the first appearance of a pink color that persists after shaking for 30 s. Add an additional 25 mL of sample, mix, and titrate with 0.02 N sodium hydroxide until the pink color is restored.
  
  **Acceptance criteria:** NMT 0.5 mL of 0.02 N sodium hydroxide is required to restore the pink color. (NMT 0.003%)

- **ALKALINITY (AS NH₄)**
  
  **Sample:** 25 mL
  
  **Analysis:** Add 2 drops of methyl red TS to 25 mL of water, add 0.02 N sulfuric acid until a red color just appears, then add the Sample, and mix.
  
  **Acceptance criteria:** NMT 0.2 mL of 0.02 N sulfuric acid is required to restore the red color. (NMT 3 mg/kg)

- **NONVOLATILE RESIDUE**
  
  **Sample:** 125 mL (about 100 g)
  
  **Analysis:** Evaporate the Sample to dryness in a tared dish on a steam bath, dry the residue at 105° for 30 min, cool, and weigh.
  
  **Acceptance criteria:** NMT 0.003%

- **SOLUBILITY IN WATER**
  
  **Sample:** Transfer 50 mL of sample to a 100-mL glass-stoppered graduated cylinder, dilute to 100 mL with water, and mix. Place the graduated cylinder, in a water bath maintained at 10°, and allow it to stand for 30 min.
  
  **Acceptance criteria:** No haze or turbidity develops.

Isopropyl Alcohol

First Published: Prior to FCC 6
Last Revision: FCC 7, Third Supplement
2-Propanol
Isopropanol

C₃H₈O  Formula wt 60.10
CAS: [67-63-0]

Published on March 26, 2020
DESCRIPTION
Isopropyl Alcohol occurs as a clear, colorless, flammable liquid. It is miscible with water, with ethyl alcohol, with ether, and with many other organic solvents.

Function: Extraction solvent

Packaging and Storage: Store in tight containers, remote from fire.

IDENTIFICATION
- REFRACTIVE INDEX, Appendix IIB [NOTE—Use an Abbé or other refractometer of equal or greater accuracy.]
  Acceptance criteria: 1.377–1.380 at 20°

ASSAY
- PROCEDURE
  System suitability solution: USP 2-Propanol System Suitability RS
  Chromatographic system, Appendix IIA
    Mode: Gas chromatography
    Detector: Flame ionization
    Column: 60-m × 0.25-mm fused silica column with 1.4-μm film thickness of 6% cyanopropylphenyl/94% dimethylpolysiloxane stationary phase1 with a 4-mm straight liner
    Temperature: Injector: 150°
    Detector: 200°
    Column: Hold at 35° for 5 min; ramp to 45° at 1°/min; ramp to 100° at 10°/min; hold at 100° for 1 min
    Carrier gas: Helium
    Linear velocity: 35 cm/s
    Injection size: 1 μL
    Split ratio: 50:1
  System suitability
    Sample: System suitability solution
    [NOTE—Approximate relative retention times for ethyl ether, acetone, isopropyl alcohol, diisopropyl ether, 1-propanol, and 2-butanol are 0.7, 0.9, 1.0, 1.4, 1.5, and 2.0, respectively.]
    Suitability requirement 1: The relative standard deviation for the main isopropyl alcohol peak is NMT 2.0% for replicate injections.
    Suitability requirement 2: The resolution for the acetone and isopropyl alcohol peaks is NLT 2.0.
    Suitability requirement 3: The signal-to-noise ratio is NLT 10 for any of the following peaks: ethyl ether, acetone, diisopropyl ether, 1-propanol, and 2-butanol.
  Analysis: Inject the sample into the chromatograph, and record the resulting chromatogram. Determine the percentage of C₅H₁₀O present in the sample through peak area normalization:

  \[
  \text{Result} = \left( \frac{R_i}{R_T} \right) \times 100
  \]

  \(R_i\) = peak area for isopropyl alcohol
  \(R_T\) = sum of all of the peak areas

  Acceptance criteria: NLT 99.7% of C₅H₁₀O

IMPURITIES
Inorganic Impurities
- LEAD, Lead Limit Test, Atomic Absorption Spectrophotometric Graphite Furnace Method, Method I, Appendix IIIB
  Acceptance criteria: NMT 1 mg/kg

SPECIFIC TESTS
- ACIDITY (AS ACETIC ACID)
  Sample: 50 mL (about 39 g)

Analysis: Add 2 drops of phenolphthalein TS to 100 mL of water, then add 0.01 N sodium hydroxide to the first pink color that persists for at least 30 s. Add the Sample to this solution, and mix. Continue the addition of 0.01 N sodium hydroxide until the pink color is restored.
  Acceptance criteria: NMT 0.7 mL of sodium hydroxide is required to restore the pink color (NMT 10 mg/kg).

- DISTILLATION RANGE, Appendix IIB
  Acceptance criteria: Within a range of 1°, including 82.3°

- NONVOLATILE RESIDUE
  Sample: 125 mL (about 100 g)
  Analysis: Evaporate the Sample to dryness in a tared dish on a steam bath, dry the residue at 105° for 30 min, cool, and weigh.
  Acceptance criteria: NMT 10 mg/kg

- SOLUBILITY IN WATER
  Sample: 10 mL
  Analysis: Mix the Sample with 40 mL of water.
  Acceptance criteria: After 1 h, the solution is as clear as an equal volume of water.

- SPECIFIC GRAVITY: Determine by any reliable method (see General Provisions).
  Acceptance criteria: NMT 0.7840 at 25°/25° (equivalent to 0.7870 at 20°/20°)

- SUBSTANCES REDUCING PERMANGANATE
  Sample: 50 mL
  Analysis: Transfer the Sample into a 50-mL glass-stoppered cylinder, add 0.25 mL of 0.1 N potassium permanganate, mix, and allow to stand for 10 min.
  Acceptance criteria: The pink color is not entirely discharged.

- WATER, Water Determination, Appendix IIB
  Acceptance criteria: NMT 0.2%

Hydrogen Peroxide

First Published: Prior to FCC 6
H₂O₂ Formula wt 34.01
CAS: [7722-84-1]

DESCRIPTION
Hydrogen Peroxide occurs as a clear, colorless liquid. The grades of Hydrogen Peroxide suitable for food use usually have a concentration between 30% and 50%. It is miscible with water.

[NOTE—Although Hydrogen Peroxide undergoes exothermic decomposition in the presence of dirt and other foreign materials, it is safe and stable under recommended conditions of handling and storage. Information on safe handling and use may be obtained from the supplier.]

Function: Bleaching, oxidizing agent; starch modifier; antimicrobial agent

Packaging and Storage: Store in a cool place in containers with a vent in the stopper.

IDENTIFICATION
- PROCEDURE
  Sample: 1 mL
  Analysis: Shake the Sample with 10 mL of water containing 1 drop of 2 N sulfuric acid, and add 2 mL of ether. Add one drop of potassium dichromate T5.
  Acceptance criteria: An evanescent blue color is produced in the water layer that, upon agitation and standing, passes into the ether layer.

1 Restek Rtx®-1301, or equivalent. Available at www.restek.com.
ASSAY

**• PROCEDURE**

**Sample solution:** Dilute an amount of sample equivalent to 300 mg of \( \text{H}_2\text{O}_2 \) to 100 mL with water.

**Analysis:** Add 25 mL of 2 N sulfuric acid to 20.0 mL of Sample solution, and titrate with 0.1 N potassium permanganate. Each mL of 0.1 N potassium permanganate is equivalent to 1.701 mg of \( \text{H}_2\text{O}_2 \).

**Acceptance criteria:** NLT the labeled concentration or within the range stated on the label

**IMPURITIES**

**Inorganic Impurities**

**• IRON**

**Sample:** 18 mL (20 g)

**Analysis:** Evaporate the Sample to dryness with 10 mg of sodium chloride on a steam bath. Dissolve the residue in 2 mL of hydrochloric acid and dilute to 50 mL with water. Add 40 mg of ammonium persulfate crystals and 10 mL of ammonium thiocyanate \( \text{TS} \), and mix.

**Acceptance criteria:** Any red or pink color produced by the Sample does not exceed that produced by 1.0 mL of Iron Standard Solution (10 \( \mu \)g Fe) in an equal volume of solution containing the quantities of the reagents used in the test. (NMT 0.5 mg/kg)

**• LEAD, Lead Limit Test, Flame Atomic Absorption Spectrophotometric Method, Appendix IIIB**

**Analysis:** Determine as directed with the following modifications: (1) Prepare only one Diluted Standard Lead Solution by transferring 40 mL of Lead Nitrate Stock Solution into a 1000-mL volumetric flask and diluting to volume with water to obtain a solution containing 4 \( \mu \)g/mL of lead (Pb) ion; (2) Replace the first paragraph under Sample Preparation with the following: Transfer 10 g of sample, into an evaporation dish; (3) Under Procedure, determine the absorbances of the Sample Preparation and Diluted Standard Lead Solution only.

**Acceptance criteria:** The absorbance of the Sample Preparation is NMT that of the Diluted Standard Lead Solution. (NMT 4 mg/kg)

**• PHOSPHATE**

**Sample:** 400 mg

**Analysis:** Evaporate the Sample to dryness on a steam bath. Dissolve the residue in 25 mL of 0.5 N sulfuric acid, add 1 mL of a 50 mg/mL ammonium molybdate tetrahydrate solution and 1 mL of p-methylaminophenol sulfate \( \text{TS} \), and allow it to stand for 2 h. Prepare a Control using 2.0 mL of Phosphate Standard Solution (20 \( \mu \)g PO\(_4\)) (see Solutions and Indicators) in an equal volume of solution containing the quantities of the reagents used for the Sample.

**Acceptance criteria:** Any blue color produced by the Sample does not exceed that produced by the Control. (NMT 0.005%)

**• Tin**

**Aluminum chloride solution:** 8.93 mg/mL of aluminum chloride (\( \text{AlCl}_3 \cdot 6\text{H}_2\text{O} \))

**Gelatin solution:** 2 mg/mL of gelatin in boiled water that has been cooled to between 50\(^\circ\) and 60\(^\circ\). [NOTE—Prepare on the day of use.]

**Standard stock solution:** Dissolve 250.0 mg of lead-free tin foil in 10 to 15 mL of hydrochloric acid, and dilute to 250.0 mL with 1:2 hydrochloric acid.

**Standard solution:** Transfer 5.0 mL of Standard stock solution into a 100-mL volumetric flask, dilute to volume with water, and mix. Transfer 2.0 mL of this solution (100 \( \mu \)g Sn) into a 250-mL Erlenmeyer flask, and add 15 mL of water, 5 mL of nitric acid, and 2 mL of sulfuric acid.

Place a small, stemless funnel in the mouth of the flask, and heat until strong fumes of sulfuric acid evolve. Cool, add 5 mL of water, evaporate again to strong fumes, and cool. Repeat the addition of water and heating to strong fumes, then add 15 mL of water, heat to boiling, and cool. Dilute to about 35 mL with water, add 1 drop of methyl red TS and 2.0 mL of the Aluminum chloride solution, and mix. Make the solution just alkaline by adding, dropwise, ammonium hydroxide and stirring gently, then add 0.1 mL in excess. [CAUTION—To avoid dissolving the aluminum hydroxide precipitate, do not add more ammonium hydroxide than 0.1 mL in excess.] Centrifuge for about 15 min at 4000 rpm, and then decant the supernatant liquid as completely as possible without disturbing the precipitate. Dissolve the precipitate in 5 mL of 1:2 hydrochloric acid, add 1.0 mL of the Gelatin solution, and dilute to 20.0 mL with a saturated solution of aluminum chloride.

[NOTE—Prepare on the day of use.]

**Sample solution:** Transfer 9 mL (10 g) of sample into a 250-mL Erlenmeyer flask, and add 15 mL of water, 5 mL of nitric acid, and 2 mL of sulfuric acid. Mix, and heat gently on a hot plate to initiate and maintain a vigorous decomposition. When decomposition is complete, place a small, stemless funnel in the mouth of the flask, and continue as directed for the Standard solution, beginning with “and heat until strong fumes of sulfuric acid evolve.”

**Analysis:** Rinse a polarographic cell or other vessel with a portion of the Standard solution, then add a suitable volume to the cell, immerse it in a constant-temperature bath maintained at 35° ± 0.2°, and deaerate by bubbling oxygen-free nitrogen or hydrogen through the solution for at least 10 min. Insert the dropping mercury electrode of a suitable polarograph, and record the polarogram from −0.2 to −0.7 V at a sensitivity of 0.0003 \( \mu \)A/mm, using a saturated calomel reference electrode. In the same manner, record a polarogram of a portion of the Sample solution at the same current sensitivity.

**Acceptance criteria:** The height of the wave produced by the Sample solution is not greater than that produced by the Standard solution at the same half-wave potential. (NMT 10 mg/kg)

**SPECIFIC TESTS**

**• ACIDITY (as H\(_2\)SO\(_4\))**

**Sample:** 9 mL (10 g)

**Analysis:** Dilute the Sample in 90 mL of carbon dioxide-free water, add methyl red TS and titrate with 0.02 N sodium hydroxide. Perform a blank determination by repeating the preceding, omitting the addition of the Sample.

**Acceptance criteria:** The volume of sodium hydroxide solution required for titration of the Sample should not be more than 3 mL greater than the volume required for the blank titration. (NMT 0.03%)

**• RESIDUE ON EVAPORATION**

**Sample:** 25 g

**Analysis:** Evaporate the Sample to dryness in a tared porcelain or silica dish on a steam bath, and continue drying to constant weight at 105°.

**Acceptance criteria:** The weight of the residue does not exceed 1.5 mg. (NMT 0.006%)
Glycerin

**First Published:** Prior to FCC 6  
**Last Revision:** FCC 11, Second Supplement

Glycerol

C₆H₁₂O₃  
**Formula wt:** 92.09  
**INS:** 422  
**CAS:** [56-81-5]

**DESCRIPTION**  
Glycerin occurs as a clear, colorless, viscous liquid. It is hygroscopic, and its solutions are neutral. Glycerin is miscible with water and with alcohol. It is insoluble in chloroform, in ether, and in fixed and volatile oils.  
[**NOTE—**An informational GC method (not a monograph requirement) for the identification and quantification of diethylene glycol and ethylene glycol in glycerin is available for FCC users interested in testing food-grade materials for these potential adulterants. See *Diethylene Glycol and Ethylene Glycol in Glycerin, Appendix XIII*].

**Function:** Humectant; solvent; bodying agent; plasticizer

**Packaging and Storage:** Store in tight containers.

**IDENTIFICATION**

- **A. INFRARED ABSORPTION,** Spectrophotometric Identification Tests, Appendix IIIIC
  - **Reference standard:** USP Glycerin RS  
  - **Sample and standard preparation:** F  
  - **Acceptance criteria:** The spectrum of the sample exhibits maxima at the same wavelengths as those in the spectrum of the Reference standard. [**NOTE—**A very strong absorption band in the glycerin spectrum at about 10.1 µm can be useful for differentiating glycerin from diethylene glycol and ethylene glycol, which both lack this band.]

- **B. PROCEDURE**
  - **Standard solution:** 2.0 mg/mL of USP Glycerin RS and 0.050 mg/mL of USP Diethylene Glycol RS in methanol  
  - **Sample solution:** 50 mg/mL in methanol

**Chromatographic system,** Appendix II A

- **Mode:** GC
  - **Detector:** Flame-ionization
  - **Column:** 0.53-mm × 30-m fused-silica analytical; coated with 3.0-µm 6% cyanopropylphenyl–94% dimethylpolysiloxane stationary phase¹

**Temperatures**

- **Injector:** 220°  
- **Detector:** 250°  
- **Column:** See Table 1.

**Table 1**

<table>
<thead>
<tr>
<th>Initial Temperature (°C)</th>
<th>Temperature Ramp (°/min)</th>
<th>Final Temperature (°C)</th>
<th>Hold Time at Final Temperature (min)</th>
</tr>
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<td>100</td>
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</tr>
<tr>
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<td>120</td>
<td>10</td>
</tr>
<tr>
<td>120</td>
<td>50</td>
<td>220</td>
<td>6</td>
</tr>
</tbody>
</table>

**Carrier gas:** Helium  
**Injection volume:** 1.0 µL  
**Flow rate:** 4.5 mL/min  
**Injection type:** Split flow ratio is about 10:1  
**System suitability**  
**Sample:** Standard solution

¹ DB-624 (J & W Scientific), or equivalent.

**ASSAY**

- **PROCEDURE**
  - **Sodium periodate solution:** Dissolve 60 g of sodium metaperiodate (NaIO₄) in sufficient water containing 120 mL of 0.1 N sulfuric acid to make 1000 mL. Do not heat to dissolve the periodate. If the solution is not clear, pass through a sintered-glass filter. Store the solution in a glass-stoppered, light-resistant container. Test the suitability of this solution as follows: Pipet 10 mL into a 250-mL volumetric flask, dilute to volume, and mix. Dissolve about 550 mg of sample in 50 mL of water, and add 50 mL of the diluted periodate solution by pipet. For a blank, pipet 50 mL of the diluted periodate solution into a flask containing 50 mL of water. Allow the solutions to stand for 30 min, then add 5 mL of hydrochloric acid and 10 mL of potassium iodide TS to each, and rotate to mix. Allow to stand for 5 min, add 100 mL of water, and titrate with 0.1 N sodium thiosulfate, shaking continuously and adding starch TS near the endpoint. The ratio of the volume of 0.1 N sodium thiosulfate required for the sample:periodate mixture to that required for the blank should be between 0.750 and 0.765.
  - **Sample:** 400 mg  
  - **Analysis:** Transfer the Sample into a 600-mL beaker, dilute with 50 mL of water, add bromothymol blue TS, and acidify with 0.2 N sulfuric acid to a definite green or green-yellow color. Neutralize with 0.05 N sodium hydroxide to a definite blue endpoint free of green color. Prepare a blank containing 50 mL of water, and neutralize in the same manner. Pipet 50 mL of the Sodium periodate solution into each beaker, mix by swirling gently, cover with a watch glass, and allow to stand for 30 min at room temperature (not above 35°) in the dark or in subdued light. Add 10 mL of a mixture consisting of equal volumes of ethylene glycol and water to each beaker, and titrate with 0.1 N sodium thiosulfate, shaking continuously and adding starch TS near the endpoint. Allow to stand for 20 min. Dilute each solution to about 300 mL with water, and titrate with 0.1 N sodium hydroxide to a pH of 8.1 ± 0.1 for the Sample and 6.5 ± 0.1 for the blank, using a pH meter previously calibrated with pH 4.0 Acid Phthalate Standard Buffer Solution (see Solutions and Indicators). Each mL of 0.1 N sodium hydroxide, after correction for the blank, is equivalent to 9.210 mg of glycerin (C₆H₁₂O₃).
  - **Acceptance criteria:** 99.0%–101.0% of glycerin (C₆H₁₂O₃) on the as-is basis

**IMPURITIES**

- **Inorganic impurities**
  - **LEAD,** Lead Limit Test, Atomic Absorption Spectrophotometric Graphite Furnace Method, Method I, Appendix IX B
  - **Acceptance criteria:** NMT 1 mg/kg

- **Organic impurities**
  - **FATTY ACIDS AND ESTERS**
    - **Sample:** 40.0 mL (50 g)  
    - **Analysis:** Mix the Sample with 50 mL of recently boiled water and 5.0 mL of 0.5 N sodium hydroxide. Boil the mixture for 5 min, cool, add phenolphthalein TS, and titrate the excess alkali with 0.5 N hydrochloric acid.
**Acceptance criteria:** NMT 1 mL of 0.5 N sodium hydroxide is consumed.

**SPECIFIC TESTS**

• **CHLORINATED COMPOUNDS (AS Cl)**

  Sample: 5.0 g
  
  **Analysis:** Transfer the Sample into a dry, 100-mL round-bottom, ground-joint flask, and add 15 mL of morpholine to it. Connect the flask with a ground joint reflux condenser, and reflux the mixture gently for 3 h. Rinse the condenser with 10 mL of water, receiving the washing into the flask, and cautiously acidify with nitric acid. Transfer the solution to a suitable comparison tube, add 0.5 mL of silver nitrate TS, dilute to 50.0 mL, and mix thoroughly.
  
  **Control:** 150 μg of chloride in an equal volume of solution containing the quantities of reagents used in the Analysis, but omitting the refluxing.
  
  **Acceptance criteria:** Any turbidity produced by the Sample does not exceed that produced by the Control. (NMT 0.003% as Cl)

• **COLOR**

  Sample: 50 mL
  
  **Control:** 0.40 mL of ferric chloride CS diluted with water to 50 mL
  
  **Analysis:** Transfer the Sample and the Control to separate 50-mL Nessler tubes of the same diameter and color and view the tubes downward against a white surface.
  
  **Acceptance criteria:** The color of the Sample is not darker than that of the Control.

• **READILY CARBONIZABLE SUBSTANCES, Appendix IIB**

  Sample: 5 mL
  
  **Analysis:** Rinse a glass-stoppered 25-mL cylinder with 95% sulfuric acid, and allow it to drain for 10 min. Add the Sample and 5 mL of 95% sulfuric acid, gently mix for 1 min at 18°–20°, and allow to stand for 1 h.
  
  **Acceptance criteria:** The resulting mixture has no more color than Matching Fluid H.

• **RESIDUE ON IGNITION**

  Sample: 50 g
  
  **Analysis:** Heat the Sample in a tared, open dish, and ignite the vapors, allowing them to burn until the sample has been completely consumed. After cooling, moisten the residue with 0.5 mL of sulfuric acid, and complete the ignition by heating for 15-min periods at 800 ± 25° to constant weight.
  
  **Acceptance criteria:** NMT 0.01%

• **SPECIFIC GRAVITY:** Determine by any reliable method (see General Provisions).
  
  **Acceptance criteria:** NLT 1.259

• **WATER, Water Determination, Method I, Appendix IIB**
  
  **Acceptance criteria:** NMT 1.0%
FCC Solutions

STANDARD BUFFER SOLUTIONS

Reagent Solutions Before mixing, dry the crystalline reagents, except the boric acid, at 110° to 120°, and use water that has been previously boiled and cooled in preparing the solutions. Store the prepared reagent solutions in chemically resistant glass or polyethylene bottles, and use within 3 months. Discard if molding is evident.

Potassium Chloride, 0.2 M Dissolve 14.91 g of potassium chloride (KCl) in sufficient water to make 1000.0 mL.

Potassium Biphthalate, 0.2 M Dissolve 40.84 g of potassium biphthalate \([KHC_6H_4(COO)_2]\) in sufficient water to make 1000.0 mL.

Potassium Phosphate, Monobasic, 0.2 M Dissolve 27.22 g of monobasic potassium phosphate \((KH_2PO_4)\) in sufficient water to make 1000.0 mL.

Boric Acid–Potassium Chloride, 0.2 M Dissolve 12.37 g of boric acid \((H_3BO_3)\) and 14.91 g of potassium chloride \((KCl)\) in sufficient water to make 1000.0 mL.

Hydrochloric Acid, 0.2 M, and Sodium Hydroxide, 0.2 M Prepare and standardize as directed under Volumetric Solutions in this section.

Procedure To prepare 200 mL of a standard buffer solution having a pH within the range 1.2 to 10.0, place 50.0 mL of the appropriate 0.2 M salt solution, prepared as above, in a 200-mL volumetric flask, add the volume of 0.2 M hydrochloric acid or of sodium hydroxide specified for the desired pH in the accompanying table, dilute with water to volume, and mix.

STANDARD SOLUTIONS FOR THE PREPARATION OF CONTROLS AND STANDARDS

The following solutions are used in tests for impurities that require the comparison of the color or turbidity produced in a solution of the test substance with that produced by a known amount of the impurity in a control. Directions for the preparation of other standard solutions are given in the monographs or under the general tests in which they are required (see also Index).

Ammonium Standard Solution (10 µg NH₄ in 1 mL) Dissolve 296.0 mg of ammonium chloride \((NH_4Cl)\) in sufficient water to make 1000.0 mL.

Barium Standard Solution (100 µg Ba in 1 mL) Dissolve 177.9 mg of barium chloride \((BaCl_2 \cdot 2H_2O)\) in water in a 1000-mL volumetric flask, dilute with water to volume, and mix.

Iron Standard Solution (10 µg Fe in 1 mL) Dissolve 702.2 mg of ferrous ammonium sulfate \([Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O]\) in 10 mL of 2 N sulfuric acid in a 100-mL volumetric

Composition of Standard Buffer Solutions

<table>
<thead>
<tr>
<th>pH</th>
<th>0.2 M HCl (mL)</th>
<th>pH</th>
<th>0.2 M HCl (mL)</th>
<th>pH</th>
<th>0.2 M HCl (mL)</th>
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<td>—</td>
<td>—</td>
<td>—</td>
<td>7.8</td>
<td>44.5</td>
</tr>
</tbody>
</table>

Dilute all final solutions to 200.0 mL (see Procedure). The standard pH values given in this table are considered to be reproducible to within ±0.02 of the pH unit specified at 25°.
flask, dilute with water to volume, and mix. Transfer 10.0 mL of this solution into a 1000-mL volumetric flask, add 10 mL of 2 N sulfuric acid, dilute with water to volume, and mix.

**Magnesium Standard Solution** (50 µg Mg in 1 mL)
Dissolve 50.0 mg of magnesium metal (Mg) in 1 mL of hydrochloric acid in a 1000-mL volumetric flask, dilute with water to volume, and mix.

**Phosphate Standard Solution** (10 µg PO₄ in 1 mL)
Dissolve 143.3 mg of monobasic potassium phosphate (KH₂PO₄) in water in a 100-mL volumetric flask, dilute with water to volume, and mix. Transfer 10.0 mL of this solution into a 1000-mL volumetric flask, dilute with water to volume, and mix.

### TEST SOLUTIONS (TS) AND OTHER REAGENTS

Certain of the following test solutions are intended for use as acid-base indicators in volumetric analyses. Such solutions should be adjusted so that when 0.15 mL of the indicator solution is added to 25 mL of carbon dioxide-free water, 0.25 mL of 0.02 N acid or alkali, respectively, will produce the characteristic color change.

In general, the directive to prepare a solution “fresh” indicates that the solution is of limited stability and must be prepared on the day of use.

**Acetic Acid** Use ACS reagent-grade Acetic Acid, Glacial (99.7% of CH₃COOH; approximately 17.5 N).

**Acetic Acid TS, Diluted** (1 N) A solution containing about 6% (w/v) of CH₃COOH. Prepare by diluting 60.0 mL of glacial acetic acid, or 166.6 mL of 36% acetic acid (6 N), with sufficient water to make 1000 mL.

**Acetic Acid TS, Strong** (5 N) A solution containing 30% (v/v) of CH₃COOH. Prepare by diluting 300.0 mL of glacial acetic acid with sufficient water to make 1000 mL.

**Acetic Periodic Acid TS** Dissolve 2.7 g of periodic acid (H₅IO₆) in 50 mL of water, add 950 mL of glacial acetic acid, and mix thoroughly. [CAUTION—This solution is an oxidizing agent and is dangerous in contact with organic materials. Do not use cork or rubber stoppers on storage bottles.]

**Alcohol** (Ethanol; Ethyl Alcohol; C₂H₅OH) Use ACS reagent-grade Ethyl Alcohol (NLT 95.0%, by volume, of C₂H₅OH).

**Alcohol, Absolute** (Anhydrous Alcohol; Dehydrated Alcohol) Use ACS reagent-grade Ethyl Alcohol, Absolute (NLT 99.5%, by volume, of C₂H₅OH).

**Alcohol, Dibutyl** A solution containing 41.0%–42.0%, by weight, corresponding to 48.4%–49.5%, by volume, at 15.56°, of C₂H₅OH.

**Alcohol, 70%** (at 15.56°) A 38.6:15 mixture (v/v) of 95% alcohol and water, having a specific gravity of 0.884 at 25°. To prepare 100 mL, dilute 73.7 mL of alcohol to 100 mL with water at 25°.

**Alcohol, 80%** (at 15.56°) A 45.5:9.5 mixture (v/v) of 95% alcohol and water, having a specific gravity of 0.857 at 25°. To prepare 100 mL, dilute 84.3 mL of alcohol to 100 mL with water at 25°.

**Alcohol, 90%** (at 15.56°) A 51:3 mixture (v/v) of 95% alcohol and water, having a specific gravity of 0.827 at 25°. To prepare 100 mL, dilute 94.8 mL of alcohol to 100 mL with water at 25°.

**Alcohol, Aldehyde-Free** Dissolve 2.5 g of lead acetate in 5 mL of water, add the solution to 1000 mL of alcohol contained in a glass-stoppered bottle, and mix. Dissolve 5 g of potassium hydroxide in 25 mL of warm alcohol, cool, and add slowly, without stirring, to the alcoholic solution of lead acetate. Allow to stand for 1 h, then shake the mixture vigorously, allow to stand overnight, decant the clear liquid, and recover the alcohol by distillation. *Ethyl Alcohol FCC, Alcohol USP*, or USSD #3A or #30 may be used. If the titration of a 250-mL sample of the alcohol by Hydroxylamine Hydrochloride TS does not exceed 0.25 mL of 0.5 N alcoholic potassium hydroxide, the above treatment may be omitted.

**Alcoholic Potassium Hydroxide TS** See *Potassium Hydroxide TS, Alcoholic*.

**Ammonia TS** (Fehling’s Solution) See Cupric Tartrate TS, Alkaline.

**Ammoniacal Silver Nitrate TS** Add 6 N ammonium hydroxide, dropwise, to a 1:20 solution of silver nitrate until the precipitate that first forms is almost, but not entirely, dissolved. Filter the solution, and place in a dark bottle.

**Ammonium Acetate TS** Dissolve 2.5 g of lead acetate in 5 mL of water, add 950 mL of glacial acetic acid, and mix thoroughly. [CAUTION—This solution is an oxidizing agent and is dangerous in contact with organic materials. Do not use cork or rubber stoppers on storage bottles.]

**Ammonium Carbonate TS** Dissolve 20 g of ammonium carbonate and 20 mL of Ammonia TS in sufficient water to make 100 mL.

**Ammonium Chloride TS** Dissolve 10.5 g of ammonium chloride (NH₄Cl) in sufficient water to make 100 mL.

**Ammonium Molybdate TS** Dissolve 6.5 g of finely powdered molybdic acid (85%) in a mixture of 14 mL of water and 14.5 mL of ammonium hydroxide. Cool the solution, and add it slowly, with stirring, to a well-cooled mixture of 32 mL of nitric acid and 40 mL of water. Allow to stand for 48 h, and pass through a fine-porosity, sintered-glass crucible lined at the bottom with a layer of glass wool. This solution deteriorates upon standing and is unsuitable for use if, upon the addition of 2 mL of Sodium Phosphate TS to 5 mL of the solution, an abundant yellow precipitate does not form at once or after slight warming. Store it in the dark. If a precipitate forms during storage, use only the clear, supernatant solution.
**Ammonium Oxalate TS** Dissolve 3.5 g of ammonium oxalate ([NH₄]₂C₂O₄ · H₂O) in sufficient water to make 100 mL.

**Ammonium Sulfanilate TS** To 2.5 g of sulfanilic acid add 15 mL of water and 3 mL of 6 N ammonium hydroxide, and mix. Add, with stirring, more 6 N ammonium hydroxide, if necessary, until the acid dissolves, adjust the pH of the solution to about 4.5 with 2.7 N hydrochloric acid, using Bromocresol Green TS as an outside indicator, and dilute to 25 mL.

**Ammonium Sulfide TS** Saturate 6 N ammonium hydroxide with hydrogen sulfide (H₂S), and add two-thirds of its volume of 6 N ammonium hydroxide. Residue upon ignition: NMT 0.05%. The solution is not rendered turbid either by Magnesium Sulfate TS or by Calcium Chloride TS (carbonate). This solution is unsuitable for use if an abundant precipitate of sulfur is present. Store in small, well-filled, dark amber-colored bottles in a cold, dark place.

**Ammonium Thiocyanate TS** (1 N) Dissolve 8 g of ammonium thiocyanate (NH₄SCN) in sufficient water to make 100 mL.

**Anthrone TS** Carefully dissolve about 0.1 g of anthrone in 100 g of sulfuric acid. Use a freshly prepared solution.

**Antimony Trichloride TS** Dissolve 20 g of antimony trichloride (SbCl₃) in chloroform to make 100 mL. Filter if necessary.

**Barium Chloride TS** Dissolve 12 g of barium chloride (BaCl₂ · 2H₂O) in sufficient water to make 100 mL.

**Barium Dihyphenylamine Sulfonate TS** Dissolve 300 mg of p-dihyphenylamine sulfonic acid barium salt in 100 mL of water.

**Barium Hydroxide TS** Use a saturated solution of barium hydroxide in recently boiled water. Use a freshly prepared solution.

**Benedict’s Qualitative Reagent** See Cupric Citrate TS, Alkaline.

**Benzedine TS** Dissolve 50 mg of benzidine in 10 mL of glacial acetic acid, dilute with water to 100 mL, and mix.

**Bismuth Nitrate TS** Reflux 5 g of bismuth nitrate [Bi(NO₃)₃ · 5H₂O] with 7.5 mL of nitric acid and 10 mL of water until dissolved, cool, filter, and dilute with water to 250 mL.

**Bromine TS** (Bromine Water) Prepare a saturated solution of bromine by agitating 2–3 mL of bromine (Br₂) with 100 mL of cold water in a glass-stoppered bottle, the stopper of which should be lubricated with petroleum. Store it in a cold place protected from light.

**Bromocresol Blue TS** Use Bromocresol Green TS.

**Bromocresol Green TS** Dissolve 50 mg of bromocresol green in 100 mL of alcohol, and filter if necessary.

**Bromocresol Purple TS** Dissolve 250 mg of bromocresol purple in 20 mL of 0.05 N sodium hydroxide, and dilute with water to 250 mL.

**Bromophenol Blue TS** Dissolve 100 mg of bromophenol blue in 100 mL of 1:2 alcohol, and filter if necessary.

**Bromothymol Blue TS** Dissolve 100 mg of bromothymol blue in 100 mL of 1:2 alcohol, and filter if necessary.

**Calcium Chloride TS** Dissolve 7.5 g of calcium chloride (CaCl₂ · 2H₂O) in sufficient water to make 100 mL.

**Calcium Hydroxide TS** A solution containing approximately 140 mg of Ca(OH)₂ in each 100 mL. To prepare, add 3 g of calcium hydroxide [Ca(OH)₂] to 1000 mL of water, and agitate the mixture vigorously and repeatedly for 1 h. Allow the excess calcium hydroxide to settle, and decant or draw off the clear, supernatant liquid.

**Calcium Sulfate TS** A saturated solution of calcium sulfate in water.

**Carr-Price Reagent** See Antimony Trichloride TS.

**Ceric Ammonium Nitrate TS** Dissolve 6.25 g of ceric ammonium nitrate [(NH₄)₂Ce(NO₃)₆] in 100 mL of 0.25 N nitric acid. Prepare the solution fresh every third day.

**Chlorine TS** (Chlorine Water) A saturated solution of chlorine in water. Place the solution in small, completely filled, light-resistant containers. Chlorine TS, even when kept from light and air, is apt to deteriorate. Store it in a cold, dark place. For full strength, prepare this solution fresh.

**Chromotropic Acid TS** Dissolve 50 mg of chromotropic acid or its sodium salt in 100 mL of 75% sulfuric acid (made by cautiously adding 75 mL of 95%–98% sulfuric acid to 33.3 mL of water).

**Cobaltous Chloride TS** Dissolve 2 g of cobaltous chloride (CoCl₂ · 6H₂O) in 1 mL of hydrochloric acid and sufficient water to make 100 mL.

**Cobalt–Uranyl Acetate TS** Dissolve, with warming, 40 g of uranyl acetate [UO₂(C₂H₃O₂)₂ · 2H₂O] in a mixture of 30 g of glacial acetic acid and sufficient water to make 500 mL. Similarly, prepare a solution containing 200 g of cobaltous acetate [Co(C₂H₃O₂)₄ · 4H₂O] in a mixture of 30 g of glacial acetic acid and sufficient water to make 500 mL. Mix the two solutions while still warm, and cool to 20°. Maintain the temperature at 20° for about 2 h to separate the excess salts from solution, and then pass through a dry filter.

**Congo Red TS** Dissolve 500 mg of congo red in a mixture of 10 mL of alcohol and 90 mL of water.

**Copper Sulfate TS** Dissolve 12.5 g of cupric sulfate in sufficient water to make 100 mL.

**Cresol Red TS** Triturate 100 mg of cresol red in a mortar with 26.2 mL of 0.01 N sodium hydroxide until solution is complete, then dilute the solution with water to 250 mL.

**Cresol Red–Thymol Blue TS** Add 15 mL of Thymol Blue TS to 5 mL of Cresol Red TS, and mix.

**Crystal Violet TS** Dissolve 100 mg of crystal violet in 10 mL of glacial acetic acid.

**Cupric Citrate TS, Alkaline** (Benedict’s Qualitative Reagent) With the aid of heat, dissolve 173 g of sodium citrate (Na₃C₆H₅O₇ · 2H₂O) and 117 g of sodium carbonate (Na₂CO₃ · H₂O) in about 700 mL of water, and filter through paper, if necessary. In a separate container, dissolve 17.3 g of cupric sulfate (CuSO₄ · 5H₂O) in about 100 mL of water, and slowly add this solution, with constant stirring, to the first solution. Cool the mixture, dilute to 1000 mL, and mix.

**Cupric Nitrate TS** Dissolve 2.4 g of cupric nitrate [Cu(NO₃)₂ · 3H₂O] in sufficient water to make 100 mL.

**Cupric Sulfate TS** Dissolve 12.5 g of cupric sulfate (CuSO₄ · 5H₂O) in sufficient water to make 100 mL, and mix.

**Cuprous Tartrate TS, Alkaline** (Fehling’s Solution) The Copper Solution (A): Dissolve 34.66 g of carefully selected, small crystals of cupric sulfate (CuSO₄ · 5H₂O) showing no trace of efflorescence or of adhering moisture, in sufficient water to make 500 mL. Store this solution in small, tight containers. The Alkaline Tartrate Solution (B): Dissolve 173 g of crystallized potassium sodium tartrate (KNaC₄H₄O₆ · 4H₂O) and 50 g of sodium hydroxide (NaOH) in sufficient water to make 500 mL. Store this solution in small, alkali-resistant containers. For use, mix exactly equal volumes of solutions A and B at the time required.

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Cyanogen Bromide TS Dissolve 5 g of cyanogen bromide in water to make 50 mL.

[Caution—Prepare this solution in a hood, as cyanogen bromide volatilizes at room temperature, and the vapor is highly irritating and poisonous.]

Deniges’ Reagent See Merccuric Sulfate TS.

Dichloro phenol-Indophenol TS Warm 100 mg of 2,6-dichlorophenol-indophenol sodium with 100 mL of water. Filter and use within 3 days.

2,7-Dihydroxynaphthalene TS Dissolve 100 mg of 2,7-dihydroxynaphthalene in 1000 mL of sulfuric acid, and allow the solution to stand until the initial color disappears. If the solution is very dark, discard it and prepare a new solution from a different supply of sulfuric acid. This solution is stable for approximately 1 month if stored in a dark bottle.

Diphenylamine TS Dissolve 1 g of diphenylamine in 100 mL of sulfuric acid. The solution should be colorless.

Diphenylcarbazone TS Dissolve about 1 g of diphenylcarbazone (C₁₀H₁₂N₂O₂) in sufficient alcohol to make 100 mL. Store this solution in a brown bottle.

Diphenylcarbazone Alcoholic TS Dissolve 100 mg of 2,7-dihydroxynaphthalene TS in 100 mL of alcohol.

Dithizone TS Dissolve 25.6 mg of dithizone in 100 mL of alcohol.

Dichloro phenol–indophenol sodium with 100 mL of water.

Dissolve 9 g of ferric chloride (FeCl₃·6H₂O) in about 100 mL of recently boiled and thoroughly cooled water. Prepare this solution fresh.

Ferrous Sulfate TS Dissolve 8 g of clear crystals of ferrous sulfate (FeSO₄·7H₂O) in about 100 mL of recently boiled and thoroughly cooled water. Prepare this solution fresh.

Formaldehyde TS A solution containing approximately 37.0% (w/v) of HCHO. It may contain methanol to prevent polymerization.

Fuchsir–Sulfurous Acid TS Dissolve 200 mg of basic fuchsin in 120 mL of hot water, and allow the solution to cool. Add a solution of 2 g of anhydrous sodium sulfite in 20 mL of water, and then add 2 mL of hydrochloric acid. Dilute the solution with water to 200 mL, and allow to stand for at least 1 h. Prepare this solution fresh.

Hydrochloric Acid Use ACS reagent-grade Hydrochloric Acid (36.5%–38.0% of HCl; approximately 12 N).

Hydrochloric Acid TS, Diluted (2.7 N) A solution containing 10% (w/v) of HCl. Prepare by diluting 226 mL of hydrochloric acid (36%) with sufficient water to make 1000 mL.

Hydrogen Peroxide A solution containing 2.5–3.5 g of H₂O₂ in each 100 mL. It may contain suitable preservatives, totaling not more than 0.05%.

Hydrogen Sulfite A saturated solution of hydrogen sulfite made by passing H₂S into cold water. Store it in small, dark, amber-colored bottles, filled nearly to the top. It is unsuitable unless it possesses a strong odor of H₂S, and unless it produces at once a copious precipitate of sulfur when added to an equal volume of Ferric Chloride TS. Store in a cold, dark place.

Hydroxylamine Hydrochloride TS Dissolve 3.5 g of hydroxyxylamine hydrochloride (NH₂OH·HCl) in 95 mL of 60% alcohol, and add 0.5 mL of a 1:1000 solution of bromophenol blue and 0.5 N alcoholic potassium hydroxide until a green tint develops in the solution. Then add sufficient 60% alcohol to make 100 mL.

Hydroxyquinoline TS Dissolve 5 g of 8-hydroxyquinoline (oxine) in sufficient alcohol to make 100 mL.

Indigo Carmine TS (Sodium Indigoindisulfonate TS) Dissolve a quantity of sodium indigoindisulfonate, equivalent to 180 mg of C₁₄H₉N₃O₄S₄Na₂, in sufficient water to make 100 mL. Use within 60 days.

Iodine TS Dissolve 14 g of iodine (I₂) in a solution of 36 g of potassium iodide (KI) in 100 mL of water, and add 3 drops of hydrochloric acid, and 3 drops of hydroxylamine hydrochloride (NH₂OH·HCl) in sufficient alcohol to make 100 mL. Add this solution to 100 mL of a 60% solution of zinc chloride, ZnCl₂, in water (sp. gr. 1.8). Keep a few crystals of iodine in the solution.

Isopropanol Use ACS reagent-grade Isopropanol Alcohol.

Isopropanol, Anhydrous (Dehydrated Isopropanol) Use isopropanol that has been previously dried by shaking with anhydrous calcium chloride, followed by filtering.

Lead Acetate TS Dissolve 9.5 g of clear, transparent crystals of lead acetate [Pb(C₁₂H₁₄O₄)₂·3H₂O] in sufficient recently boiled water to make 100 mL. Store in well-stoppered bottles.

Lead Subacetate TS Triturate 14 g of lead monoxide (PbO) to a smooth paste with 10 mL of water, and transfer.

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the mixture to a bottle, using an additional 10 mL of water for rinsing. Dissolve 22 g of lead acetate [Pb(C₂H₃O₂)₂ · 3H₂O] in 70 mL of water, and add the solution to the lead oxide mixture. Shake it vigorously for 5 min, then set it aside, shaking it frequently during 7 days. Finally, filter, and add enough recently boiled water through the filter to make 100 mL.

**Lead Subacetate TS, Diluted** Dilute 3.25 mL of Lead Subacetate TS with sufficient water, recently boiled and cooled, to make 100 mL. Store in small, well-fitted, tight containers.

**Litmus TS** Digest 25 g of powdered litmus with three successive 100-mL portions of boiling alcohol, continuing each extraction for about 1 h. Filter, wash with alcohol, and discard the alcohol filtrate. Macerate the residue with about 25 mL of cold water for 4 h, filter, and discard the filtrate. Finally, digest the residue with 125 mL of boiling water for 1 h, cool, and filter.

**Magnesia Mixture TS** Dissolve 5.5 g of magnesium chloride (MgCl₂ · 6H₂O) and 7 g of ammonium chloride (NH₄Cl) in 65 mL of water, add 35 mL of 6 N ammonium hydroxide, set the mixture aside for a few days in a well-stopped bottle, and filter. If the solution is not perfectly clear, filter it before using.

**Magnesium Sulfate TS** Dissolve 12 g of crystals of magnesium sulfate (MgSO₄ · 7H₂O), selected for freedom from efflorescence, in water to make 100 mL.

**Malachite Green TS** Dissolve 1 g of malachite green oxalate in 100 mL of glacial acetic acid.

**Mayer’s Reagent** See Mercuric–Potassium Iodide TS.

**Mercuric Acetate TS** Dissolve 6 g of mercuric acetate [Hg(C₂H₃O₂)₂] in sufficient glacial acetic acid to make 100 mL. Store in tight containers protected from direct sunlight.

**Mercuric Chloride TS** Dissolve 6.5 g of mercuric chloride (HgCl₂) in water to make 100 mL.

**Mercuric–Potassium Iodide TS (Mayer’s Reagent)** Dissolve 1.358 g of mercuric chloride (HgCl₂) in 60 mL of water. Dissolve 5 g of potassium iodide (KI) in 10 mL of water. Mix the two solutions, and add water to make 100 mL.

**Mercuric–Potassium Iodide TS, Alkaline (Nessler’s Reagent)** Dissolve 10 g of potassium iodide (KI) in 10 mL of water, and add slowly, with stirring, a saturated solution of mercuric chloride until a slight red precipitate remains undissolved. To this mixture add an ice-cold solution of 30 g of potassium hydroxide (KOH) in 60 mL of water, then add 1 mL more of the saturated solution of mercuric chloride. Dilute with water to 200 mL. Allow the precipitate to settle, and draw off the clear liquid. A 2-mL portion of this reagent, when added to 100 mL of a 1:300,000 solution of ammonium chloride in ammonia-free water, instantly produces a yellow-brown color.

**Mercuric Sulfate TS (Denigès’ Reagent)** Mix 5 g of yellow mercuric oxide (HgO) with 40 mL of water, and while stirring, slowly add 20 mL of sulfuric acid, then add another 40 mL of water, and stir until completely dissolved.

**Mercuric Nitrate TS** Dissolve 15 g of mercuric nitrate in a mixture of 90 mL of water and 10 mL of 2 N nitric acid. Store in dark, amber-colored bottles in which a small globule of mercury has been placed.

**Methanol (Methyl Alcohol)** Use ACS reagent-grade Methanol.

**Methanol, Anhydrous (Dehydrated Methanol)** Use Methanol.

**p-Methylaminophenol Sulfate TS** Dissolve 2 g of p-methylaminophenol sulfate [(HOCH₂NHCH₃)₂ · H₂SO₄] in 100 mL of water. To 10 mL of this solution add 90 mL of water and 20 g of sodium bisulfite. Confirm the suitability of this solution by the following test: Add 1 mL of the solution to each of four tubes containing 25 mL of 0.5 N sulfuric acid and 1 mL of Ammonium Molybdate TS. Add 5 µg of phosphorus (PO₄) to one tube, 10 µg to a second, and 20 µg to a third, using 0.5 mL, 1.0 mL, and 2.0 mL, respectively, of Phosphate Standard Solution, and allow to stand for 2 h. The solutions in the three tubes should show readily perceptible differences in blue color corresponding to the relative amounts of phosphorus added, and the one to which 5 µg of phosphorus was added should be perceptibly bluer than the blank.

**Methylene Blue TS** Dissolve 125 mg of methylene blue in 100 mL of alcohol, and dilute with alcohol to 250 mL.

**Methyl Orange TS** Dissolve 100 mg of methyl orange in 100 mL of water, and filter if necessary.

**Methyl Red TS** Dissolve 100 mg of methyl red in 100 mL of alcohol, and filter if necessary.

**Methyl Red–Methylene Blue TS** Add 10 mL of Methyl Red TS to 10 mL of Methylene Blue TS, and mix.

**Methylrosaniline Chloride TS** See Crystal Violet TS.

**Methyl Violet TS** See Crystal Violet TS.

**Millon’s Reagent** To 2 mL of mercury in an Erlenmeyer flask add 20 mL of nitric acid. Shake the flask in a hood to break the mercury into small globules. After about 10 min add 35 mL of water, and if a precipitate or crystals appear, add sufficient 1:5 nitric acid (prepared from nitric acid from which the oxides have been removed by blowing air through it until it is colorless) to dissolve the separated solid. Add a 1:10 solution of sodium hydroxide, dropwise, with thorough mixing, until the curdy precipitate that forms after the addition of each drop no longer redissolves but is dispersed to form a suspension. Add 5 mL more of the dilute nitric acid, and mix well. Prepare this solution fresh.

**α-Naphtholbenzoxide TS** Dissolve 0.2 g of α-naphtholbenzoxide in glacial acetic acid to make 100 mL. Sensitivity: Add 100 mL of freshly boiled and cooled water to 0.2 mL of a 1:1000 solution of α-naphtholbenzoxide in ethanol, and add 0.1 mL of 0.1 N sodium hydroxide: a green color develops. Add subsequently 0.2 mL of 0.1 N hydrochloric acid: the color of the solution changes to yellow-red.

**Naphthol Green TS** Dissolve 500 mg of naphthol green B in water to make 1000 mL.

**Nessler’s Reagent** See Alkaline Mercuric–Potassium Iodide TS.

**Neutral Red TS** Dissolve 100 mg of neutral red in 100 mL of 50% alcohol.

**Nickel Standard Solution TS (10 mg/kg)** Prepare a 0.40% (w/v) solution of analytical reagent-grade nickel chloride (NiCl₂ · 6H₂O) with water. Pipet 1.0 mL of the solution into a 100-mL volumetric flask, and dilute with water to volume.

**Ninhydrin TS** See Triketohydridine Hydrate TS.

**Nitric Acid** Use ACS reagent-grade Nitric Acid (approximately 15.7 N).

**Nitric Acid TS, Diluted (1.7 N)** A solution containing about 10% (w/v) of HNO₃. Prepare by diluting 105 mL of nitric acid (70%) with water to make 1000 mL.

**Orthophenanthroline TS** Dissolve 150 mg of orthophenanthroline (C₁₂H₈N₂ · H₂O) in 10 mL of a solution of ferrous sulfate, prepared by dissolving 700 mg of clear
crystals of ferrous sulfate (\(\text{FeSO}_4 \cdot 7\text{H}_2\text{O}\)) in 100 mL of water. The ferrous sulfate solution must be prepared immediately before dissolving the orthophenanthroline. Store the solution in well-closed containers.

**Oxalic Acid TS**  Dissolve 6.3 g of oxalic acid (\(\text{H}_2\text{C}_2\text{O}_4 \cdot 2\text{H}_2\text{O}\)) in water to make 100 mL.

**Phenol Red TS** (Phenolsulfonphthalein TS) Dissolve 100 mg of phenolsulfonphthalein in 100 mL of alcohol, and filter if necessary.

**Phenolphthalein TS** Dissolve 1 g of phenolphthalein in 100 mL of alcohol.

**Phenolsulfonphthalein TS** See Phenol Red TS.

**p-Phenylphenol TS** On the day of use, dissolve 750 mg of \(p\)-phenylphenol in 50 mL of Sodium Hydroxide TS.

**Phosphoric Acid** Use ACS reagent-grade Phosphoric Acid (NLT 85.0% of \(\text{H}_3\text{PO}_4\)).

**Phosphotungstic Acid TS** Dissolve 1 g of phosphotungstic acid (approximately 24\(\text{WO}_3 \cdot 2\text{H}_2\text{PO}_4 \cdot 48\text{H}_2\text{O}\)) in water to make 100 mL.

**Picric Acid TS** See Trinitrophenol TS.

**Potassium Acetate TS** Dissolve 10 g of potassium acetate (\(\text{KC}_2\text{H}_3\text{O}_2\)) in water to make 100 mL.

**Potassium Chromate TS** Dissolve 10 g of potassium chromate (\(\text{K}_2\text{Cr}_2\text{O}_7\)) in water to make 100 mL.

**Potassium Dichromate TS** Dissolve 7.5 g of potassium dichromate (\(\text{K}_2\text{Cr}_2\text{O}_7\)) in water to make 100 mL.

**Potassium Ferricyanide TS** (10%) Dissolve 1 g of potassium ferricyanide \([\text{K}_3\text{Fe(CN)}_6]\) in 10 mL of water. Prepare this solution fresh.

**Potassium Ferrocyanide TS** Dissolve 1 g of potassium ferrocyanide \([\text{K}_3\text{Fe(CN)}_6 \cdot 3\text{H}_2\text{O}]\) in 10 mL of water. Prepare this solution fresh.

**Potassium Hydroxide TS** (1 N) Dissolve 6.5 g of potassium hydroxide (KOH) in water to make 100 mL.

**Potassium Hydroxide TS, Alcoholic** Use 0.5 N Alcoholic Potassium Hydroxide (see Volumetric Solutions in this section).

**Potassium Iodide TS** Dissolve 16.5 g of potassium iodide (KI) in water to make 100 mL. Store in light-tight containers.

**Potassium Permanganate TS** Use 0.1 N Potassium Permanganate (see Volumetric Solutions in this section).

**Potassium Pyroantimonate TS** Dissolve 1 g of potassium pyroantimonate in 95 mL of hot water. Cool quickly, and add a solution containing 2.5 g of potassium hydroxide in 50 mL of water and 1 mL of an 8.5:100 solution of sodium hydroxide. Allow to stand for 24 h, filter, and dilute with water to 150 mL.

**Potassium Sulfate TS** Dissolve 1 g of potassium sulfate \((\text{K}_2\text{SO}_4)\) in sufficient water to make 100 mL.

**Quimociac TS** Dissolve 70 g of sodium molybdate \((\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O})\) in 150 mL of water (Solution A). Dissolve 60 g of citric acid in a mixture of 85 mL of nitric acid and 150 mL of water, and cool (Solution B). Gradually add Solution A to Solution B, with stirring, to produce Solution C. Dissolve 5.0 mL of natural or synthetic quimociac in a mixture of 35 mL of nitric acid and 100 mL of water (Solution D). Gradually add Solution D to Solution C, mix well, and allow to stand overnight. Filter the mixture, add 280 mL of acetone to the filtrate, dilute with water to 1000 mL, and mix. Store in a polyethylene bottle.

**Starch TS** Mix 1 g of a suitable starch and sufficient cold water to make a thin paste. Add 20 mL of boiling water, boil for 1 min with continuous stirring, and cool. Use only the clear solution. Test the sensitivity of the Starch TS as follows: Prepare a solution of 50 mg/kg chlorine by diluting 1 mL of a commercial 5% sodium hypochlorite (\(\text{NaOCl}\)) solution in 1000 mL of water. Combine 5 mL of Starch TS with 100 mL of water and add 0.5 mL of 0.1 N potassium iodide. Addition of one drop of the 50 mg/kg chlorine solution should give a swirl of color where the drop hits. Addition of 1 mL of 50 mg/kg chlorine solution should give a deep blue color throughout the solution. The deep blue color produced is discharged by addition of 0.05 mL of 0.1 N Potassium Iodide TS. Dissolve 16.5 g of potassium iodide \((\text{KI})\) in water to make 100 mL. Prepare this solution fresh.

**Silver Nitrate TS** Use 0.1 N Silver Nitrate (see Volumetric Solutions in this section).

**Sodium Bisulfite TS** Dissolve 10 g of sodium bisulfite \((\text{NaHSO}_3)\) in water to make 30 mL. Prepare this solution fresh.

**Sodium Bitartrate TS** Dissolve 2 g of sodium bitartrate \((\text{Na}_2\text{C}_4\text{H}_4\text{O}_6 \cdot 10\text{H}_2\text{O})\) in water to make 100 mL.

**Sodium Carbonate TS** Dissolve 10.6 g of each anhydrous sodium carbonate \((\text{Na}_2\text{CO}_3)\) in water to make 100 mL.

**Sodium Cobaltinitrite TS** Dissolve 10 g of sodium cobaltinitrite \([\text{Na}_3\text{Co(NO)}_2\text{Cl}_4]\) in water to make 50 mL, and filter if necessary.

**Sodium Fluoride TS** Dry about 500 mg of sodium fluoride (NaF) at 200° for 4 h. Weigh accurately 222 mg of the dried sodium fluoride, and dissolve it in sufficient water to make exactly 100 mL. Transfer 10.0 mL of this solution into a 1000-mL volumetric flask, dilute with water to volume, and mix. Each mL of this final solution corresponds to 10 μg of fluoride (F).

**Sodium Hydroxide TS** (1 N) Dissolve 4.3 g of sodium hydroxide \((\text{NaOH})\) in water to make 100 mL.

**Sodium Indigotindisulfonate TS** See Indigo Carmine TS.

**Sodium Nitroferricyanide TS** Dissolve 1 g of sodium nitroferricyanide \([\text{Na}_3\text{Fe(NO)}_2\text{CN)}_3 \cdot 2\text{H}_2\text{O}]\) in water to make 20 mL. Prepare this solution fresh.

**Sodium Phosphate TS** Dissolve 12 g of clear crystals of dibasic sodium phosphate \((\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O})\) in water to make 100 mL.

**Sodium Sulfite TS** Dissolve 1 g of sodium sulfite \((\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O})\) in water to make 10 mL. Prepare this solution fresh.

**Sodium Tetraphenylborate TS** Dissolve 1.2 g of sodium tetraphenylborate in water to make 200 mL. If necessary, stir for 5 min with 1 g of freshly prepared hydrous aluminum oxide, and filter to clarify.

**Sodium Thiosulfate TS** Use 0.1 N Sodium Thiosulfate (see Volumetric Solutions in this section).

**Stannous Chloride TS** Dissolve 40 g of reagent-grade stannous chloride dihydrate \((\text{SnCl}_2 \cdot 2\text{H}_2\text{O})\) in 100 mL of hydrochloric acid.

**Starch TS** Mix 1 g of a suitable starch and sufficient cold water to make a thin paste. Add 20 mL of boiling water, boil for 1 min with continuous stirring, and cool. Use only the clear solution. Test the sensitivity of the Starch TS as follows: Prepare a solution of 50 mg/kg chlorine by diluting 1 mL of a commercial 5% sodium hypochlorite (\(\text{NaOCl}\)) solution in 1000 mL of water. Combine 5 mL of Starch TS with 100 mL of water and add 0.5 mL of 0.1 N potassium iodide. Addition of one drop of the 50 mg/kg chlorine solution should give a swirl of color where the drop hits. Addition of 1 mL of 50 mg/kg chlorine solution should give a deep blue color throughout the solution. The deep blue color produced is discharged by addition of 0.05 mL of 0.1 N Potassium Iodide TS. Dissolve 6.3 g of oxalic acid (\(\text{H}_2\text{C}_2\text{O}_4 \cdot 2\text{H}_2\text{O}\)) in 100 mL of water. The oxalic acid solution must be prepared immediately before dissolving the orthophenanthroline. Store the solution in well-closed containers.

**Quinidine Red TS** Dissolve 100 mg of quinidine red in 100 mL of glacial acetic acid.

**Schiff’s Reagent, Modified** Dissolve 200 mg of rosaniline hydrochloride (\(\text{C}_{30}\text{H}_{39}\text{ClN}_3\)) in 120 mL of hot water. Cool, add 2 g of sodium bisulfite \((\text{NaHSO}_3)\) followed by 2 mL of hydrochloric acid, and dilute with water to 200 mL. Store in a brown bottle at 15° or lower.
sodium thiosulfate. Prepare fresh solution when Starch TS no longer passes the sensitivity test.

**Starch Iodide Paste TS** Heat 100 mL of water in a 250-mL beaker to boiling, add a solution of 750 mg of potassium iodide (KI) in 5 mL of water, then add 2 g of zinc chloride (ZnCl₂) dissolved in 10 mL of water, and while the solution is boiling, add with stirring a smooth suspension of 5 g of potato starch in 30 mL of cold water. Continue to boil for 2 min, then cool. Store in well-closed containers in a cool place. This mixture must show a definite blue streak when a glass rod dipped in a mixture of 1 mL of 0.1 M sodium nitrite, 500 mL of water, and 10 mL of hydrochloric acid is streaked on a smear of the paste.

**Sulfanilic Acid TS** Dissolve 800 mg of sulfanilic acid (p-NH₂C₆H₄SO₃H · H₂O) in 100 mL of acetic acid. Store in tight containers.

**Sulfuric Acid** Use ACS reagent-grade Sulfuric Acid (95.0%–98.0% of H₂SO₄; approximately 36 N).

**Sulfuric Acid TS** (95%) Add a quantity of sulfuric acid of known concentration to sufficient water to adjust the final concentration to 94.5%–95.5% of H₂SO₄. Because the acid concentration may change upon standing or upon intermittent use, the concentration should be checked frequently and solutions assaying more than 95.5% or less than 94.5% discarded or adjusted by adding either diluted or fuming sulfuric acid, as required.

**Sulfuric Acid TS, Diluted (2 N)** A solution containing 10% (w/v) of H₂SO₄. Prepare by cautiously adding 57 mL of sulfuric acid (95%–98%) or Sulfuric Acid TS to about 100 mL of water, then cool to room temperature, and dilute with water to 1000 mL.

**Tannic Acid TS** Dissolve 1 g of tannic acid (tannin) in 1 mL of alcohol, and add water to make 10 mL. Prepare this solution fresh.

**Thymol Blue TS** Dissolve 100 mg of thymol blue in 100 mL of alcohol, and filter if necessary.

**Thymolphthalein TS** Dissolve 100 mg of thymolphthalein in 100 mL of alcohol, and filter if necessary.

**Triketohydrindene Hydrate TS** (Ninhydrin TS) Dissolve 200 mg of triketohydrindene hydrate (C₉H₄O₃ · H₂O) in water to make 100 mL. Prepare this solution fresh.

**Trinitrophenol TS** (Picric Acid TS) Dissolve the equivalent of 1 g of anhydrous trinitrophenol in 100 mL of hot water. Cool the solution, and filter if necessary.

**Xylenol Orange TS** Dissolve 100 mg of xylenol orange in 100 mL of alcohol.
APPENDIX II: PHYSICAL TESTS AND DETERMINATIONS

A. CHROMATOGRAPHY

[Note—Chromatographic separations may also be characterized according to the type of instrumentations or apparatus used. The types of chromatography that may be used in the Food Chemicals Codex (FCC) are column, thin-layer, gas, and high-pressure or high-performance liquid chromatography. The Committee on Food Chemicals Codex recognizes that the field of chromatography continues to advance. Accordingly, the use of equivalent or improved systems is acceptable with appropriate validation.]

For the purposes of the FCC, chromatography is defined as an analytical technique whereby a mixture of chemicals may be separated by virtue of their differential affinities for two immiscible phases. One of these, the stationary phase, consists of a fixed bed of small particles with a large surface area, while the other, the mobile phase, is a gas or liquid that moves constantly through, or over the surface of, the fixed phase. Chromatographic systems achieve their ability to separate mixtures by selectively retarding the passage of some compounds through the stationary phase while permitting others to move more freely. Therefore, the chromatogram may be evaluated qualitatively by determining the retardation factor, for each of the eluted substances. The retardation factor is defined as the ratio of the distance traveled by the solute band to the distance traveled by the mobile solvent in a particular time. The retardation factor, $R_t$, can be evaluated by the expression:

$$R_t = \frac{V_m C_m}{(V_m C_m + V_s C_s)}$$

in which $V_m$ and $V_s$ are the volumes of the mobile and stationary phase, respectively, and $C_m$ and $C_s$ are the concentrations of the solute in either phase at any time. This can be simplified to:

$$R_t = V_m/(V_m + KV_o)$$

in which $K = C_o/C_m$ and is an equilibrium constant that indicates this differential affinity of the solute for the phases. Alternatively, a new constant, $k$, the capacity factor, may be introduced, giving another form of the expression:

$$R_t = 1/(1 + k)$$

in which $k = KV_o/V_m$. The capacity factor, $k$, which is normally constant for small samples, is a parameter that expresses the ability of a particular chromatographic system to interact with a solute. The larger the $k$ value, the more the sample is retarded.

Both the retardation factor and the capacity factor may be used for qualitative identification of a solute or for developing strategies for improving separation. In terms of parameters easily obtainable from the chromatogram, the $R_t$ is defined as the ratio of the distance traveled by the solute band to the distance traveled by the mobile solvent in a particular time. The capacity factor, $k$, can be evaluated by the expression:

$$k = \frac{(t_r - t_o)}{t_o}$$

in which $t_r$, the retention time, is the elapsed time from the start of the chromatogram to the elution maximum of the solute, and $t_o$ is the retention time of a solute that is not retained by the chromatographic system.

Retardation of the solutes by the stationary phase may be achieved by one or a combination of mechanisms. Certain substances, such as alumina or silica gel, interact with the solutes primarily by adsorption, either physical adsorption, in which the binding forces are weak and easily reversible, or chemisorption, in which strong bonding to the surface can occur. Another important mechanism of retardation is partition, which occurs when the solute dissolves in the stationary phase, usually a liquid coated as a thin layer on the surface of an inert particle or chemically bonded to it. If the liquid phase is a polar substance (e.g., polyethylene glycol) and the mobile phase is nonpolar, the process is termed normal-phase chromatography. When the stationary phase is nonpolar (e.g., octadecylsilane) and the mobile phase is polar, the process is reversed-phase chromatography. For the separation of mixtures of ionic species, insoluble polymers called ion exchangers are used as the stationary phase. Ions of the solutes contained in the mobile phase are adsorbed onto the surface of the ion exchanger while at the same time displacing an electrically equivalent amount of less strongly bound ions to maintain the electroneutrality of both phases. The chromatographic separation of mixtures of large molecules such as proteins may be accomplished by a mechanism called size exclusion chromatography. The stationary phases used are highly cross-linked polymers that have imbibed a sufficient amount of solvent to form a gel. The separation is based on the physical size of the solvated solutes; those that are too large to fit within the interstices of the gel are eluted rapidly, while the smaller molecules permeate into the pores of the gel and are eluted later. In any chromatographic separation, more than one of the above mechanisms may be occurring simultaneously.

Chromatographic separations may also be characterized according to the type of instrumentation or apparatus used. The types of chromatography that may be used in the FCC are column, thin-layer, gas, and high-performance liquid chromatography.
Column Chromatography

APPARATUS

The equipment needed for column chromatography is not elaborate, consisting only of a cylindrical glass or Teflon tube that has a restricted outflow orifice. The dimensions of the tube are not critical and may vary from 10 to 40 mm in inside diameter and from 100 to 600 mm in length. For a given separation, greater efficiency may be obtained with a long narrow column, but the resultant flow rate will be lower. A fritted-glass disk may be seated in the end of the tube to act as a support for the packing material. The column is fitted at the end with a stopcock or other flow-restriction device to control the rate of delivery of the eluant.

PROCEDURE

The stationary phase is introduced into the column either as a dry powder or as a slurry in the mobile phase. Because a homogeneous bed free of void spaces is necessary to achieve maximum separation efficiency, the packing material is introduced in small portions and allowed to settle before further additions are made. Settling may be accomplished by allowing the mobile phase to flow through the bed, by tapping or vibrating the column if a dry powder is used, or by compressing each added portion using a tamping rod. The rod can be a solid glass, plastic, or metal cylinder whose diameter is slightly smaller than that of the column, or it can be a thinner rod onto the end of which has been attached a disk of suitable diameter. Ion-exchange resins and exclusion polymers are never packed as dry powders because after introduction of the mobile phase, they will swell and create sufficient pressure to shatter the column. When the packing has been completed, the sample is introduced onto the top of the column. If the sample is soluble, it is dissolved in a minimum amount of the mobile phase, pipetted onto the column, and allowed to percolate into the top of the bed. If it is not soluble or if the volume of solution is too large, it may be mixed with a small amount of the column packing. This material is then transferred to the chromatographic tube to form the top of the bed.

The chromatogram is then developed by adding the mobile phase to the column in small portions and allowing it to percolate through the packed bed either by gravity or under the influence of pressure or vacuum. Development of the chromatogram takes place by selective retardation of the components of the mixture as a result of their interaction with the stationary phase. In column chromatography, the stationary phase may act by adsorption, partition, ion exchange, exclusion of the solutes, or a combination of these effects.

When the development is complete, the components of the sample mixture may be detected and isolated by either of two procedures. The entire column may be extruded carefully from the tube, and if the compounds are colored or fluorescent under ultraviolet light, the appropriate segments may be cut from the column using a razor blade. If the components are colorless, they may be visualized by painting or spraying a thin longitudinal section of the surface of the chromatogram with color-developing reagents. The chemical may then be separated from the stationary phase by extraction with a strong solvent such as methanol and subsequently quantitated by suitable methods.

In the second procedure, the mobile phase may be allowed to flow through the column until the components of the mixture successively appear in the effluent. This eluate may be collected in fractions and the mobile phase evaporated if desired. The chemicals present in each fraction may then be determined by suitable analytical techniques.

Paper Chromatography

In this type of chromatography, the stationary phase ordinarily consists of a sheet of paper of suitable texture and thickness. The paper used is made from highly purified cellulose, which has a great affinity for water and other polar solvents since it has many hydroxyl functional groups. The tightly bound water acts as the stationary phase, and therefore the mechanism that predominates is liquid–liquid or partition chromatography. Adsorption of solutes to the cellulose surface may also occur, but this is of lesser importance. Papers especially impregnated to permit ion-exchange or reverse-phase chromatography are also available.

APPARATUS

The essential equipment for paper chromatography consists of the following:

Vapor-tight chamber: The chamber is constructed preferably of glass, stainless steel, or porcelain. It is provided with inlets for the addition of solvent or for releasing internal pressure, and it is designed to permit observation of the progress of the chromatographic run without being opened. Tall glass cylinders are convenient if they are made vapor-tight with suitable covers and a sealing compound.

Supporting rack: The rack serves as a support for the solvent troughs and antisiphoning rods. It is constructed of a corrosion-resistant material about 5 cm shorter than the inside height of the chamber.

Solvent troughs: The troughs, made of glass, are designed to be longer than the width of the chromatographic sheets and to contain a volume of solvent greater than that required for one chromatographic run.

Antisiphoning rods: Constructed of heavy glass, the rods are placed on the rack and arranged to run outside of, parallel to, and slightly above the edge of the glass trough.

Chromatographic sheets: Special chromatographic filter paper is cut to length approximately equal to the height of the chamber. The sheet is a least 2.5 cm wide but not wider than the length of the trough. A fine pencil line is drawn horizontally across the filter paper at a distance from one end such that when the sheet is suspended from the antisiphoning rods with the upper end of the paper resting in the trough and the lower portion hanging free into the chamber, the line is...
located a few cm below the rods. Care is necessary to avoid contaminating the paper by excessive handling or by contact with dirty surfaces.

**PROCEDURE FOR DESCENDING CHROMATOGRAPHY**

Separation of substances by descending chromatography is accomplished by allowing the mobile phase to flow downward on the chromographic sheet.

The substance or substances to be analyzed are dissolved in a suitable solvent. Convenient volumes of the resulting solution, normally containing 1–20 µg of the compound, are placed in 6–10-mm spots along the pencil line not less than 3 cm apart. If the total volume to be applied would produce spots of a diameter greater than 6–10 mm, it is applied in separate portions to the same spot, each portion being allowed to dry before the next is added.

The spotted chromatographic sheet is suspended in the chamber by use of the antisiphoning rod and an additional heavy glass rod that holds the upper end of the sheet in the solvent trough. The bottom of the chamber is covered with a mixture containing both phases of the prescribed solvent system. It is important to ensure that the portion of the sheet hanging below the rods is freely suspended in the chamber without touching the rack or the chamber walls. The chamber is sealed to allow equilibration (saturation) of the chamber and the paper with solvent vapor. Any excess pressure is released as necessary. For large chambers, equilibration overnight may be necessary.

A volume of the mobile phase in excess of the volume required for complete development of the chromatogram is saturated with the immobile phase. After equilibration of the chamber, the prepared mobile solvent is introduced into the trough through the inlet. The inlet is closed, and the mobile phase is allowed to travel down the paper the desired distance. Precautions must be taken against allowing the solvent to run down the sheet when opening the chamber and removing the chromatogram. The location of the solvent front is quickly marked, and the sheets are dried.

The chromatogram is observed and measured directly or after suitable development to reveal the location of the spots of the isolated components of the mixture.

**PROCEDURE FOR ASCENDING CHROMATOGRAPHY**

In ascending chromatography, the lower edge of the sheet (or strip) is dipped into the mobile phase to permit the mobile phase to rise on the chromographic sheet.

The test materials are applied to the chromatographic sheet as directed under Procedure for Descending Chromatography. Enough of both phases of the solvent mixture to cover the bottom of the chamber is added. Empty solvent troughs are placed on the bottom of the chamber, and the chromatographic sheet is suspended so that the end near which the spots have been added hangs free inside the empty trough.

The chamber is sealed, and equilibration is allowed to proceed as described under Procedure for Descending Chromatography. Then the solvent is added through the inlet to the trough in excess of the quantity of solvent required for complete moistening of the chromatographic sheet. The chamber is resealed. When the solvent front has reached the desired height, the chamber is opened and the sheet is removed, the location of the solvent front is quickly marked, and the sheet is dried.

Small cylinders may be used without troughs so that only the mobile phase is placed on the bottom. The chromatographic sheet is suspended during equilibration with the lower end just above the solvent, and chromatography is started by lowering the sheet so that it touches the solvent.

**DETECTION OF CHROMATOGRAPHIC BANDS**

After the chromatogram has been fully developed, the bands corresponding to the various solutes may be detected by means similar to those described in Column Chromatography. If the compounds are colored or fluorescent under ultraviolet light, they may be visualized directly. Colorless compounds may be detected by spraying the paper with color-developing reagents. The bands corresponding to the individual components can be cut from the paper, and the chemical substances eluted from the cellulose by the use of a strong solvent such as methanol.

**IDENTIFICATION OF SOLUTES**

Since the chromatographic mobilities of the solutes may change from run to run due to varying experimental conditions, presumptive identification of a substance should be based on comparison with a reference standard. The $R_f$ values of the unknown substance and the standard on the same chromatogram must be identical. Alternatively, the ratio between the distances traveled by a given compound and a reference substance, the $R_f$ value, must be 1.0. Identification may also be made by mixing a small amount of the reference substance with the unknown and chromatographing. The resulting chromatogram should contain only one spot. Definitive identification of solutes may be achieved by eluting them from the paper and subjecting them to IR, NMR, or mass spectrometry.

**Thin-Layer Chromatography**

In thin-layer chromatography (TLC), the stationary phase is a uniform layer of a finely divided powder that has been coated on the surface of a glass or plastic sheet and that is held in place by a binder. The capacity of the system is dependent on the thickness of the layer, which may range from 0.1–2.0 mm. The thinner layers are used primarily for analytical separations, while the thicker layers, because of their greater sample-handling ability, are useful for preparative work.
Substances that are used as coatings in TLC include silica gel, alumina, cellulose, and reversed-phase packings. Separations occur because of adsorption of the solutes from the mobile phase onto the surface of the thin layer. However, adsorption of water from the air or solvent components from the mobile phase can give rise to partition or liquid–liquid chromatography. The stationary phase of TLC plates has an average particle size of 10–15 µm, and that of high-performance TLC (HPTLC) plates has an average particle size of 5 µm. Commercial plates with a preadsorbent zone can be used if they are specified in a monograph. Sample applied to the preadsorbent region develops into sharp, narrow bands at the preadsorbent-sorbent interface. The separations achieved may be based on adsorption, partition, or a combination of both effects, depending on the particular type of stationary phase. Specially coated plates are available that permit ion-exchange or reversed-phase separations.

**APPARATUS**

Acceptable apparatus and materials for thin-layer chromatography consist of the following:

**TLC plates:** Flat plates of uniform thickness throughout their areas. Common sizes are 20 cm, 10 cm, and 5 cm × 20 cm.

**(Plates are typically glass, plastic, or metal.)**

**Aligning tray:** An aligning tray or other suitable flat surface is used to align and hold plates during application of the adsorbent.

**Adsorbent:** The adsorbent may consist of finely divided adsorbent materials for chromatography. It can be applied directly to the glass plate, or it can be bonded to the plate by means of plaster of Paris or with starch paste. Pretreated chromatographic plates are available commercially.

**Spreader:** A suitable spreading device that, when moved over the glass plate, applies a uniform layer of adsorbent of the desired thickness over the entire surface of the plate.

**Storage rack:** A rack of convenient size to hold the prepared plates during drying and transportation.

**Developing chamber:** A glass chamber that can accommodate one or more plates and can be properly closed and sealed. It is fitted with a plate-support rack that can support the plates when the lid of the chamber is in place.

[NOTE—Preformed TLC plates available commercially may also be used.]

**PROCEDURE**

A general method for preparation of TLC plates is included below. In the case of both TLC and HPTLC, commercially-prepared plates are readily available from multiple suppliers and are commonly used.

Clean the plates scrupulously, as by immersion in a chromic acid cleansing mixture, rinse them with copious quantities of water until the water runs off the plates without leaving any visible water or oily spots, and dry.

Arrange the plate or plates on the aligning tray, and secure them so that they will not slip during the application of the adsorbent. Mix an appropriate quantity of adsorbent and liquid, usually water, which when shaken for 30 s gives a smooth slurry that will spread evenly with the aid of a spreader. Transfer the slurry to the spreader, and apply the coating at once before the binder begins to harden. Move the spreader smoothly over the plates from one end of the tray to the other. Remove the spreader, and wipe away excess slurry. Allow the plates to set for 10 min, and then place them in the storage rack, and dry at 105° for 30 min or as directed in the individual monograph. Store the finished plates in a desiccator.

Equilibrate the atmosphere in the Developing Chamber by placing in it a volume of the mobile phase in excess of that required for complete development of the chromatogram, cover the chamber with its lid, and allow it to stand for at least 30 min.

The Sample Solution and the Standard Solution are spotted on the surface of the stationary phase (plate) at the prescribed volume in sufficiently small portions to obtain circular spots of 2–5 mm in diameter (1–2 mm on HPTLC plates) or bands of 10–20 mm × 1–2 mm (5–10 mm × 0.5–1 mm on HPTLC plates) at an appropriate distance from the lower edge and sides of the plate. [NOTE—During development, the application position must be at least 5 mm (TLC) or 3 mm (HPTLC) above the level of the mobile phase.] The solutions are applied on a line parallel to the lower edge of the plate with an interval of at least 10 mm (5 mm on HPTLC plates) between the centers of spots, or 4 mm (2 mm on HPTLC plates) between the edges of bands, then allowed to dry.

Arrange the plate on the supporting rack (sample spots on the bottom), and introduce the rack into the developing chamber. The solvent in the chamber must be deep enough to reach the lower edge of the adsorbent, but must not touch the spot points. Seal the cover in place, and maintain the system until the solvent ascends to a point 10–15 cm above the initial spots. Remove the plates, and dry them in air. Measure and record the distance of each spot from the point of origin. If so directed, spray the spots with the reagent specified, observe, and compare the sample with the standard chromatogram.

**DETECTION AND IDENTIFICATION**

Detection and identification of solute bands is done by methods essentially the same as those described in Column Chromatography. However, in TLC an additional method called fluorescence quenching is also used. In this procedure, an inorganic phosphor is mixed with the adsorbent before it is coated on the plate. When the developed chromatogram is irradiated with ultraviolet light, the surface of the plate fluoresces with a characteristic color, except in those places where ultraviolet-absorbing solutes are situated. These quench the fluorescence and are detectable as dark spots.

Detection with an ultraviolet light source suitable for observations with short (254-nm) and long (360-nm) ultraviolet wavelengths may be called for in some cases.
QUANTITATIVE ANALYSIS

Two methods are available if quantitation of the solute is necessary. In the first, the bands are detected and their positions marked. Those areas of adsorbent containing the compounds of interest are scraped from the surface of the plate into a centrifuge tube. The chemicals are extracted from the adsorbent with the aid of a suitable strong solvent, the suspension is centrifuged, and the supernatant layer is subjected to appropriate methods of quantitative analysis.

The second method involves the use of a scanning densitometer. This is a spectrophotometric device that directs a beam of monochromatic radiation across the surface of the plate. After interaction with the solutes in the adsorbent layer, the radiation is detected as transmitted or reflected light and a recording of light intensity versus distance traveled is produced. The concentration of a particular species is proportional to the area under its peak and can be determined accurately by comparison with standards.

Gas Chromatography

The distinguishing features of gas chromatography are a gaseous mobile phase and a solid or immobilized liquid stationary phase. Liquid stationary phases are available in packed or capillary columns. In the packed columns, the liquid phase is deposited on a finely divided, inert solid support, such as diatomaceous earth or porous polymer, which is packed into a column that typically has a 2-mm to 4-mm id and is 1–3 m long. In capillary columns, which contain no particles, the liquid phase is deposited on the inner surface of the fused silica column and may be chemically bonded to it. In gas–solid chromatography, the solid phase is an active adsorbent, such as alumina, silica, or carbon, packed into a column. Polyaromatic porous resins, which are sometimes used in packed columns, are not coated with a liquid phase.

When a volatile compound is introduced into the carrier gas and carried into the column, it is partitioned between the gas and stationary phases by a dynamic countercurrent distribution process. The compound is carried down the column by the carrier gas, retarded to a greater or lesser extent by sorption and desorption in the stationary phase. The elution of the compound is characterized by the partition ratio, \( k \), a dimensionless quantity also called the capacity factor. It is equivalent to the ratio of the time required for the compound to flow through the column (the retention time) to the retention time of a nonretarded compound. The value of the capacity factor depends on the chemical nature of the compound; the nature, amount, and surface area of the liquid phase; and the column temperature. Under a specified set of experimental conditions, a characteristic capacity factor exists for every compound. Separation by gas chromatography occurs only if the compounds concerned have different capacity factors.

APPARATUS

A gas chromatograph consists of a carrier gas source, an injection port, column, detector, and recording device. The injection port, column, and detector are carefully temperature controlled. The typical carrier gas is helium or nitrogen, depending on the column and detector in use. The gas is supplied from a high-pressure cylinder and passes through suitable pressure-reducing valves to the injection port and column. Compounds to be chromatographed, either in solution or as gases, are injected into the gas stream at the injection port. Depending on the configuration of the apparatus, the test mixture may be injected directly into the column or be vaporized in the injection port and mixed into the flowing carrier gas before entering the column.

Once in the column, compounds in the test mixture are separated by virtue of differences in their capacity factors, which in turn depend on their vapor pressure and degree of interaction with the stationary phase. The capacity factor, which governs resolution and retention times of components of the test mixture, is also temperature dependent. The use of temperature-programmable column ovens takes advantage of this dependence to achieve efficient separation of compounds differing widely in vapor pressure.

As resolved compounds emerge from the column, they pass through a detector, which responds to the amount of each compound present. The type of detector to be used depends on the nature of the compounds to be analyzed, and is specified in the individual monograph. Detectors are heated above the maximum column operating temperature to prevent condensation of the eluting compounds.

Detector output is recorded as a function of time, producing a chromatogram, which consists of a series of peaks on a time axis. Each peak represents a compound in the vaporized test mixture, although some peaks may overlap. The elution time is characteristic of the individual compounds (qualitative analysis), and the peak area is a function of the amount present (quantitative analysis).

Injectors: Sample injection devices range from simple syringes to fully programmable automatic injectors. The amount of sample that can be injected into a capillary column without overloading is small compared with the amount that can be injected into a packed column, and may be less than the smallest amount that can be manipulated satisfactorily by syringe. Capillary columns are therefore used with injectors able to split samples into two fractions, a small one that enters the column and a large one that goes to waste (split injector). Such injectors may also be used in a splitless mode for analyses of trace or minor components.

Purge and trap injectors are equipped with a sparging device by which volatile compounds in solution are carried into a low-temperature trap. When sparging is complete, trapped compounds are thermally desorbed into the carrier gas by rapid heating of the temperature-programmable trap.

Headspace injectors are equipped with a thermostatically controlled sample-heating chamber. Solid or liquid samples in tightly closed containers are heated in the chamber for a fixed period of time, allowing the volatile components in the sample to reach an equilibrium between the nongaseous phase and the gaseous or headspace phase.

After this equilibrium has been established, the injector automatically introduces a fixed amount of the headspace in the sample container into the gas chromatograph.
**Columns:** Capillary columns, which are usually made of fused silica, have a 0.2-mm to 0.53-mm id and are 5–30 m long. The liquid or stationary phase is 0.1–1.0 µm thick, although nonpolar stationary phases may be up to 5 µm thick.

Packed columns, made of glass or metal, are 1–3 m long, with a 2-mm to 4-mm id. Those used for analysis typically have liquid phase loadings of about 5% (w/w) on a solid support.

Supports for analysis of polar compounds on low-capacity, low-polarity liquid phase columns must be inert to avoid peak tailing. The reactivity of support materials can be reduced by silanizing before coating with liquid phase. Acid-washed, flux-calcined diatomaceous earth is often used for drug analysis. Support materials are available in various mesh sizes, with 80- to 100-mesh and 100- to 120-mesh being more commonly used with 2-mm to 4-mm columns. Because of the absence of a solid support, capillary compounds are much more inert than packed columns.

Retention time and the peak efficiency depend on the carrier gas flow rate; retention time is also directly proportional to column length, while resolution is proportional to the square root of the column length. For packed columns, the carrier gas flow rate is usually expressed in mL/min at atmospheric pressure and room temperature. It is measured at the detector outlet with a soap film flow meter while the column is at operating temperature. Unless otherwise specified in the individual monograph, flow rates for packed columns are 60–75 mL/min for 4-mm id columns and ~30 mL/min for 2-mm id columns.

For capillary columns, linear flow velocity is often used instead of flow rate. This is conveniently determined from the length of the column and the retention time of a dilute methane sample, provided a flame-ionization detector is in use. Typical linear velocities are 20–60 cm/s for helium. At high operating temperatures there is sufficient vapor pressure to result in a gradual loss of liquid phase, a process called “bleeding.”

**Detectors:** Flame-ionization detectors are used for most analyses, with lesser use made of thermal conductivity, electron-capture, nitrogen–phosphorus, and mass spectrometric detectors. For quantitative analyses, detectors must have a wide linear range (10³–10⁶) and are sensitive to organic compounds. Unless otherwise specified in individual monographs, flame-ionization detectors with either helium or nitrogen carrier gas are to be used for packed columns, and helium is used for capillary columns.

The thermal conductivity detector detects changes in the thermal conductivity of the gas stream as solutes are eluted. Although its linear dynamic range is smaller than that of the flame-ionization detector, it is quite rugged and occasionally used with packed columns, especially for compounds that do not respond to flame-ionization detectors.

The alkali flame-ionization detector, sometimes called an NP or nitrogen–phosphorus detector, contains a thermionic source, such as an alkali-metal salt or a glass element containing rubidium or other metal, that results in the efficient ionization of organic nitrogen and phosphorus compounds. It is a selective detector that shows little response to hydrocarbons.

The electron-capture detector contains a radioactive source (usually ⁶⁰Ni) of ionizing radiation. It exhibits an extremely high response to compounds containing halogens and nitro groups but little response to hydrocarbons. The sensitivity increases with the number and atomic weight of the halogen atoms.

**Data collection devices:** Modern data stations receive the detector output, calculate peak areas, and print chromatograms, complete with run parameters and peak data. Chromatographic data may be stored and reprocessed, with integration and other calculation variables being changed as required. Data stations are used also to program the chromatograph, controlling most operational variables and providing for long periods of unattended operation.

Data can also be collected for manual measurement on simple recorders or on integrators whose capabilities range from those providing a printout of peak areas to those providing chromatograms with peak areas and peak heights calculated and data stored for possible reprocessing.

**PROCEDURE**

Capillary columns must be tested to ensure that they comply with the manufacturers’ specifications before they are used. These tests consist of the following injections: a dilute methane sample to determine the linear flow velocity; a mixture of alkanes (e.g., C₆, C₁₃, and C₁₆) to determine resolution; and a polarity test mixture to check for active sites on the column. The latter mixture may include a methyl ester, an unsaturated compound, a phenol, an aromatic amine, a diol, a free carboxylic acid, and a polycyclic aromatic compound, depending on the samples to be analyzed.

Packed columns must be conditioned before use until the baseline and other characteristics are stable. This may be done by operation at a temperature above that called for by the method or by repeated injections of the compound or mixture to be chromatographed. A suitable test for support inertness should be done. Very polar molecules (like free fatty acids) may require a derivatization step.

Before any column is used for assay purposes, a calibration curve should be constructed to verify that the instrumental response is linear over the required range and that the curve passes through the origin. If the compound to be analyzed is adsorbed within the system, the calibration curve will intersect the abscissa at a nonzero value. This may result in error, particularly for compounds at low concentrations determined by a procedure based on a single reference point. At high concentrations, the liquid phase may be overloaded, leading to loss of peak height and symmetry.

Assays require quantitative comparison of one chromatogram with another. A major source of error is irreproducibility in the amount of sample injected, notably when manual injections are made with a syringe. The effects of variability can be minimized by the addition of an internal standard, a noninterfering compound present at the same concentration as in the sample and standard solutions. The ratio of peak response of the analyte to that of the internal standard is compared from one chromatogram to another. Where the internal standard is chemically similar to the substance being determined, there is also compensation for minor variations in column and detector characteristics. In some cases, the internal standard may be carried through the sample preparation procedure before gas chromatography to control other quantitative aspects of the assay. Automatic injectors greatly improve the reproducibility of sample injections and reduce the need for internal standards.

Many monographs require that system suitability requirements be met before samples are analyzed, see System Suitability below.
High-Performance Liquid Chromatography

High-performance liquid chromatography (HPLC) is a separation technique based on a solid stationary phase and a liquid mobile phase. Separations are achieved by partition, adsorption, exclusion, or ion-exchange processes, depending on the type of stationary phase used. HPLC has distinct advantages over gas chromatography for the analysis of nonvolatile organic compounds. Compounds to be analyzed are dissolved in a liquid, and most separations take place at room temperature.

As in gas chromatography, the elution time of a compound can be described by the capacity factor, κ, which depends on the chemical nature of the composition and flow rate of the mobile phase, and the composition and surface area of the stationary phase. Column length is an important determinant of resolution. Only compounds having different capacity factors can be separated by HPLC.

APPARATUS

A liquid chromatograph consists of one, two, or more reservoirs containing the mobile phase, a pump to force the mobile phase through the system at high pressure, an injector to introduce the sample into the mobile phase, a chromatographic column, a detector, and a data collection device such as a computer, integrator, or recorder. Short, 3-cm, 5-cm, 10-cm, and 25-cm small-bore columns containing densely packed particles of stationary phase provide for the rapid exchange of compounds between the mobile and stationary phases. In addition to receiving and reporting detector output, computers used to control chromatographic settings and operations, thus providing for long periods of unattended operation.

Pumping systems: HPLC pumping systems deliver metered amounts of mobile phase from the solvent reservoirs to the column through high-pressure tubing and fittings. Modern systems consist of one or more computer-controlled metering pumps that can be programmed to vary the ratio of mobile phase components, as required for gradient chromatography, or to mix isocratic mobile phases (i.e., mobile phases having a fixed ratio of solvents). However, the proportion of ingredients in premixed isocratic mobile phases can be more accurately controlled than in those delivered by most pumping systems. Operating pressures up to 5000 psi with delivery rates up to about 10 mL/min are typical. Pumps used for quantitative analysis should be constructed of materials inert to corrosive mobile phase components and be capable of delivering the mobile phase at a constant rate with minimal fluctuations over extended periods of time.

Injectors: After dissolution in mobile phase or other suitable solution, compounds to be chromatographed are injected into the mobile phase, either manually by syringe or loop injectors, or automatically by autosamplers. The latter consist of a carousel or rack to hold sample vials with tops that have a pierceable septum or stopper and an injection device to transfer sample from the vials to a calibrated, fixed-volume loop from which it is loaded into the chromatograph. Some autosamplers can be programmed to control sample volume, the number of injections and loop rinse cycles, the interval between injections, and other operating variables.

Some valve systems incorporate a calibrated sample loop that is filled with test solution for transfer to the column in the mobile phase. In other systems, test solution is transferred to a cavity by syringe and then switched into the mobile phase.

Columns: For most analyses, separation is achieved by partition of compounds in the test solution between the mobile and stationary phases. Systems consisting of polar stationary phases and nonpolar mobile phases are described as normal phase, while the opposite arrangement, polar mobile phases and nonpolar stationary phases, is called reversed-phase chromatography. Partition chromatography is almost always used for hydrocarbon-soluble compounds of a molecular weight that is less than 1000. The affinity of a compound for the stationary phase, and thus its retention time on the column, is controlled by making the mobile phase more or less polar. Mobile phase polarity can be varied by the addition of a second, and sometimes a third or even a fourth, component.

Stationary phases for modern, reversed-phase liquid chromatography typically consist of an organic phase chemically bound to silica or other materials. Particles are usually 3 μm, 5 μm, or 10 μm in diameter, but sizes may range up to 50 μm for preparative columns. Small particles thinly coated with organic phase allow fast mass transfer and, hence, rapid transfer of compounds between the stationary and mobile phases. Column polarity depends on the polarity of the bound functional groups, which range from relatively nonpolar octadecyl silane to very polar nitrile groups.

Columns used for analytical separations usually have internal diameters of 2–4.6 mm; larger diameter columns are used for preparative chromatography. Columns may be heated to give more efficient separations, but only rarely are they used at temperatures above 60° because of potential stationary phase degradation or mobile phase volatility. Unless otherwise specified in the individual monograph, columns are used at an ambient temperature.

Ion-exchange chromatography is used to separate water-soluble, ionizable compounds of molecular weights that are less than 2000. The stationary phases are usually synthetic organic resins; cation-exchange resins contain negatively charged active sites and are used to separate basic substances such as amines, while anion-exchange resins have positively charged active sites for separation of compounds with negatively charged groups such as phosphate, sulfonate, or carboxylate groups. Water-soluble ionic or ionizable compounds are attracted to the resins, and differences in affinity bring about the chromatographic separation. The pH of the mobile phase, temperature, ion type, ionic concentration, and organic modifiers affect the equilibrium, and these variables can be adjusted to obtain the desired degree of separation.

In size-exclusion chromatography, columns are packed with a porous stationary phase. Molecules of the compounds being chromatographed are filtered according to size. Those too large to enter the pores pass unretained through the column (total exclusion). Smaller molecules enter the pores and are increasingly retained as molecular size decreases. These columns are typically used to remove high molecular weight matrices or to characterize the molecular weight distribution of a polymer.

Detectors: Many compendial HPLC methods require the use of spectrophotometric detectors. Such a detector consists of a flow-through cell mounted at the end of the column. A beam of ultraviolet radiation passes through the flow cell and into the detector. As compounds elute from the column, they pass through the cell and absorb the radiation, resulting in measurable energy level changes.

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Fixed, variable, and photodiode array (PDA) detectors are widely available. Fixed wavelength detectors operate at a single wavelength, typically 254 nm, emitted by a low-pressure mercury lamp. Variable wavelength detectors contain a continuous source, such as a deuterium or high-pressure xenon lamp, and a monochromator or an interference filter to generate monochromatic radiation at a wavelength selected by the operator. Modern variable wavelength detectors can be programmed to change wavelength while an analysis is in progress. Multi-wavelength detectors measure absorbance at two or more wavelengths simultaneously. In diode array multi-wavelength detectors, continuous radiation is passed through the sample cell, then resolved into its constituent wavelengths, which are individually detected by the photodiode array. These detectors acquire absorbance data over the entire UV-visible range, thus providing the analyst with chromatograms at multiple, selectable wavelengths and spectra of the eluting peaks. Diode array detectors usually have lower signal-to-noise ratios than fixed or variable wavelength detectors, and thus are less suitable for analysis of compounds present at low concentrations.

Differential refractometer detectors measure the difference between the refractive index of the mobile phase alone and that of the mobile phase containing chromatographed compounds as it emerges from the column. Refractive index detectors are used to detect non-UV absorbing compounds, but they are less sensitive than UV detectors. They are sensitive to small changes in solvent composition, flow rate, and temperature, so that a reference column may be required to obtain a satisfactory baseline.

Fluorometric detectors are sensitive to compounds that are inherently fluorescent or that can be converted to fluorescent derivatives either by chemical transformation of the compound or by coupling with fluorescent reagents at specific functional groups. If derivatization is required, it can be done before chromatographic separation or, alternatively, the reagent can be introduced into the mobile phase just before its entering the detector.

Electrochemical detectors with carbon-paste electrodes may be used advantageously to measure nanogram quantities of easily oxidized compounds, notably phenols and catechols.

**Data collection devices:** Modern data stations receive and store detector output and print out chromatograms complete with peak heights, peak areas, sample identification, and method variables. They are also used to program the liquid chromatograph, controlling most variables and providing for long periods of unattended operation.

Data also may be collected on simple recorders for manual measurement or on stand-alone integrators, which range in complexity, from those providing a printout of peak areas to those providing a printout of peak areas and peak heights calculated and data stored for possible subsequent reprocessing.

**PROCEDURE**

The mobile phase composition significantly influences chromatographic performance and the resolution of compounds in the mixture being chromatographed. Composition has a much greater effect than temperature on the capacity factor, k.

In partition chromatography, the partition coefficient, and hence the separation, can be changed by addition of another component to the mobile phase. In ion-exchange chromatography, pH and ionic strength as well as changes in the composition of the mobile phase affect capacity factors. The technique of continuously increasing mobile phase strength during the chromatographic run is called gradient elution or solvent programming. It is sometimes used to chromatograph complex mixtures of components differing greatly in their capacity factors. Detectors that are sensitive to change in solvent composition, such as the differential refractometer, are more difficult to use with the gradient elution technique.

For accurate quantitative work, high-purity, “HPLC-grade” solvents and reagents must be used. The detector must have a broad linear dynamic range, and compounds to be measured must be resolved from any interfering substances. The linear dynamic range of a compound is the range over which the detector signal response is directly proportional to the amount of the compound. For maximum flexibility in quantitative work, this range should be about three orders of magnitude. HPLC systems are calibrated by plotting peak responses in comparison with known concentrations of a reference standard, using either an external or an internal standardization procedure.

Reliable quantitative results are obtained by external calibration if automatic injectors or autosamplers are used. This method involves direct comparison of the peak responses obtained by separately chromatographing the test and reference standard solutions. If syringe injection, which is irreproducible at the high pressures involved, must be used, better quantitative results are obtained by the internal calibration procedure where a known amount of a noninterfering compound, the internal standard, is added to the test and reference standard solutions, and the ratios of peak responses of the analyte and internal standard are compared.

Because of normal variations in equipment, supplies, and techniques, a system suitability test is required to ensure that a given operating system may be generally applicable. The main features of System Suitability tests are described below. For information on the interpretation of results, see the section Interpretation of Chromatograms.

**INTERPRETATION OF CHROMATOGRAMS**

*Figure 1* represents a typical chromatographic separation of two substances, 1 and 2, in which \( t_{R1} \) and \( t_{R2} \) are the respective retention times; \( h \), \( h/2 \), and \( w_{h/2} \) are the height, the half-height, and the width at half-height, respectively, for peak 1; and \( w_1 \) and \( w_2 \) are the respective widths of peaks 1 and 2 at the baseline. Air peaks are a feature of gas chromatograms and correspond to the solvent front in liquid chromatography.
Chromatographic retention times are characteristic of the compounds they represent but are not unique. Coincidence of retention times of a test and a reference substance can be used as a feature in construction of an identity profile but is insufficient on its own to establish identity. Absolute retention times of a given compound vary from one chromatogram to the next. Comparisons are normally made in terms of relative retention, which is calculated by the equation:

$$\alpha = \frac{t_{R(2)} - t_{R(0)}}{t_{R(1)} - t_{O}}$$

in which \(t_{R(2)}\) and \(t_{R(1)}\) are the retention times, measured from the point of injection, of the test and reference substances, respectively, determined under identical experimental conditions on the same column, and \(t_{O}\) is the retention time of a nonretained substance, such as methane in this case, of gas chromatography.

In this and the following expressions, the corresponding retention volumes or linear separations on the chromatogram, both of which are directly proportional to retention time, may be substituted in the equations. Where the value of \(t_{O}\) is small, \(R_r\) may be estimated from the retention times measured from the point of injection \(t_{R(2)}/t_{R(1)}\).

The number of theoretical plates, \(N\), is a measure of column efficiency. For Gaussian peaks, it is calculated by the equations:

$$N = 16\left(\frac{t_{R}}{W}\right)^2 \quad \text{or} \quad N = 5.54\left(\frac{t_{R}}{W_{1/2}}\right)^2$$

in which \(t_{R}\) is the retention time of the substance and \(W\) is the width of the peak at its base, obtained by extrapolating the relatively straight sides of the peak to the baseline. \(W_{1/2}\) is the peak width at half-height, obtained directly by electronic integrators. The value of \(N\) depends on the substance being chromatographed as well as the operating conditions such as mobile phase or carrier gas flow rates and temperature, the quality of the packing, the uniformity of the packing within the column, and for capillary columns, the thickness of the stationary phase film and the internal diameter and length of the column.

The separation of two components in a mixture, the resolution, \(R\), is determined by the equation:

$$R = \frac{2(t_{R(2)} - t_{R(1)})}{W_{2} + W_{1}}$$

in which \(t_{R(2)}\) and \(t_{R(1)}\) are the retention times of the two components, and \(W_{2}\) and \(W_{1}\) are the corresponding widths at the bases of the peaks obtained by extrapolating the relatively straight sides of the peaks to the baseline.

Peak areas and peak heights are usually proportional to the quantity of compound eluting. These are commonly measured by electronic integrators but may be determined by more classical approaches. Peak areas are generally used but may be less accurate if peak interference occurs. For manual measurements, the chart should be run faster than usual, or a comparator should be used to measure the width at half-height and the width at the base of the peak, to minimize error in these measurements. For accurate quantitative work, the components to be measured should be separated from any interfering components. Peak tailing and fronting and the measurement of peaks on solvent tails are to be avoided (see Figure 2). The relative standard deviation is expressed by the equation:

$$S_{R}^2 = \left\{ \frac{100}{X} \left[ \sum_{i=1}^{n} \left( X_i - \bar{X} \right)^2 \right] / (N-1) \right\}^{1/2}$$

in which \(S_{R}\) is the relative standard deviation in percent, \(\bar{X}\) is the mean of the set of \(N\) measurements, and \(X_i\) is an individual measurement. When an internal standard is used, the measurement \(X_i\) usually refers to the measurement of relative area, \(A_i\):

$$X_i = A_i = a_i / a$$

in which \(a\) is the area of the peak corresponding to the standard substance and \(a_i\) is the area of the peak corresponding to the internal standard. When peak heights are used, the measurement \(X_i\) refers to the measurement of relative heights, \(H_i\):

$$X_i = H_i = h_i / h$$

in which \(h\) is the height of the peak corresponding to the standard substance and \(h_i\) is the height of the peak corresponding to the internal standard.
SYSTEM SUITABILITY

Such tests are an integral part of gas and liquid chromatographic methods. They are used to verify that the resolution and reproducibility of the chromatographic system are adequate for the analysis to be done. The tests are based on the concept that the equipment, electronics, analytical operations, and samples to be analyzed constitute an integral system that can be evaluated as such.

The resolution, $R$, is a function of column efficiency, $N$, and is specified to ensure that closely eluting compounds are resolved from each other, to establish the general resolving power of the system, and to ensure that internal standards are resolved from the analyte. Column efficiency may be specified also as a system suitability requirement, especially if there is only one peak of interest in the chromatogram; however, it is a less reliable means to ensure resolution than direct measurement. Column efficiency is a measure of peak sharpness, which is important for the detection of trace components.

Replicate injections of a standard preparation used in the assay or other standard solution are compared to ascertain whether requirements for precision are met. Unless otherwise specified in the individual monograph, data from five replicate injections of the analyte are used to calculate the relative standard deviation if the requirement is 2.0% or less; data from six replicate injections are used if the relative standard deviation requirement is more than 2.0%.

The tailing factor, $T$, a measure of peak symmetry, is unity for perfectly symmetrical peaks, and its value increases as tailing becomes more pronounced. In some cases, values less than unity may be observed. As peak asymmetry increases, integration, and hence precision, becomes less reliable. The calculation is expressed by the equation:

$$\text{tailing factor} = T = \frac{W_{0.05}}{2f}$$

These tests are performed by collecting data from replicate injections of standard or other solutions as specified in the individual monograph. The specification of definitive parameters in a monograph does not preclude the use of other suitable operating conditions (see Procedures under Tests and Assays in General Provisions). Adjustments of operating conditions to meet system suitability requirements may be necessary.

Unless otherwise directed in the monograph, system suitability parameters are determined from the analyte peak. To ascertain the effectiveness of the final operating system, it should be subjected to a suitability test before use and during testing whenever there is a significant change in equipment or in a critical reagent or when a malfunction is suspected.

B. PHYSICOCHEMICAL PROPERTIES

Distillation Range

SCOPE

This method is to be used for determining the distillation range of pure or nearly pure compounds or mixtures having a relatively narrow distillation range of about 40° or less. The result so determined is an indication of purity, not necessarily of identity. Products having a distillation range of greater than 40° may be determined by this method if a wide-range thermometer, such as ASTM E1, 1C, 2C, or 3C, is specified in the individual monograph.

DEFINITIONS

**Distillation range:** The difference between the temperature observed at the start of a distillation and that observed at which a specified volume has distilled, or at which the dry point is reached.

**Initial boiling point:** The temperature indicated by the distillation thermometer at the instant the first drop of condensate leaves the end of the condenser tube.

**Dry point:** The temperature indicated at the instant the last drop of liquid evaporates from the lowest point in the distillation flask, disregarding any liquid on the side of the flask.
APPARATUS

Distillation flask: A 200-mL round-bottom distilling flask of heat-resistant glass is preferred when sufficient sample (in excess of 100 mL) is available for the test. If a sample of less than 100 mL must be used, a smaller flask having a capacity of at least double the volume of the liquid taken may be employed. The 200-mL flask has a total length of 17–19 cm, and the inside diameter of the neck is 20–22 mm. Attached about midway on the neck, approximately 12 cm from the bottom of the flask, is a side arm 10–12.7 cm long and 5 mm in internal diameter, which forms an angle of 70°–75° with the lower portion of the neck.

Condenser: Use a straight glass condenser of heat-resistant tubing, 56–60 cm long and equipped with a water jacket so that about 40 cm of the tubing is in contact with the cooling medium. The lower end of the condenser may be bent to provide a delivery tube or it may be connected to a bent adapter that serves as the delivery tube.

[Note—All glass apparatus with standard-taper ground joints may be used alternatively if the assembly employed provides results equal to those obtained with the flask and condenser described above.]

Receiver: The receiver is a 100-mL cylinder that is graduated in 1-mL subdivisions and calibrated “to contain.” It is used for measuring the sample as well as for receiving the distillate.

Thermometer: An accurately standardized partial-immersion thermometer having the smallest practical subdivisions (not greater than 0.2°) is recommended to avoid the necessity for an emergent stem correction. Suitable thermometers are available as the ASTM E1 Series 37C through 41C, and 102C through 107C, or as the MCA types R-1 through R-4 (see Thermometers, Appendix I).

Source of heat: A Bunsen burner is the preferred source of heat. An electric heater may be used, however, if it is shown to give results comparable to those obtained with the gas burner.

Shield: The entire burner and flask assembly should be protected from external air currents. Any efficient shield may be employed for this purpose.

Flask support: A heat-resistant board, 5–7 mm in thickness and having a 10-cm circular hole, is placed on a suitable ring or platform support and fitted loosely inside the shield to ensure that hot gases from the source of heat do not come in contact with the sides or neck of the flask. A second 5–7-mm thick heat-resistant board, 14–16-cm square and provided with a 30–40-mm circular hole, is placed on top of the first board. This board is used to hold the 200-mL distillation flask, which should be fitted firmly on the board so that direct heat is applied to the flask only through the opening in the board.

PROCEDURE

[Note—For materials boiling below 50°, cool the liquid to below 10° before sampling, receive the distillate in a water bath cooled to below 10°, and use water cooled to below 10° in the condenser.]

Measure 100 ± 0.5 mL of the liquid in the 100-mL graduate, and transfer the sample, together with an efficient antibumping device, into the distilling flask. Do not use a funnel in the transfer or allow any of the sample to enter the side arm of the flask. Place the flask on the heat-resistant boards, which are supported on a ring or platform, and position the shield for the flask and burner. Connect the flask and condenser, place the graduate under the outlet of the condenser tube, and insert the thermometer. The thermometer should be located in the center of the neck so that the top of the contraction chamber (or bulb, if 37C or 38C is used) is level with the bottom of the outlet to the side arm. Regulate the heating so that the first drop of liquid is collected within 5–10 min. Read the thermometer at the instant the first drop of distillate falls from the end of the condenser tube, and record as the initial boiling point. Continue the distillation at the rate of 4 or 5 mL/min of distillate, noting the temperature as soon as the last drop of liquid evaporates from the bottom of the flask (dry point) or when the specified percentage has distilled over. Correct the observed temperature readings for any variation in the barometric pressure from the normal (760 mm) by allowing 0.1° for each 2.7 mm of variation, adding the correction if the pressure is lower, or subtracting if higher, than 760 mm.

When a total-immersion thermometer is used, correct for the temperature of the emergent stem:

\[
\text{Result} = 0.00015 \times N(T - t)
\]

in which N represents the number of degrees of emergent stem from the bottom of the stopper, T represents the observed temperatures of the distillation, and t represents the temperature registered by an auxiliary thermometer, the bulb of which is placed midway of the emergent stem, adding the correction to the observed readings of the main thermometer.

Melting Range or Temperature Determination

For purposes of the FCC, the melting range or temperature of a solid is defined as those points of temperature within which or the point at which the solid coalesces and is completely melted when determined as directed below. Any apparatus or method capable of equal accuracy may be used. The accuracy should be checked frequently by the use of one or more of the six USP Melting Point Reference Standards, preferably the one that melts nearest the melting temperature of the compound to be tested.

Five procedures for the determination of melting range or temperature are given herein, varying in accordance with the nature of the substance. When no class is designated in the monograph, use the procedure for Class I.

The procedure known as the mixed melting point determination, whereby the melting range of a solid under test is compared with that of an intimate mixture of equal parts of the solid and an authentic specimen of it, may be used as a confirmatory identification test. Agreement of the observations on the original and the mixture usually constitutes reliable evidence of chemical identity.
APPARATUS

The melting range apparatus consists of a glass container for a bath of colorless fluid, a suitable stirring device, an accurate thermometer (see Appendix I), and a controlled source of heat. The bath fluid is selected consistent with the temperature required, but light paraffin is used generally, and certain liquid silicones are well adapted to the higher temperature ranges. The fluid is deep enough to permit immersion of the thermometer to its specified immersion depth so that the bulb is still about 2 cm above the bottom of the bath. The heat may be supplied electrically or by an open flame. The capillary tube is about 10 cm long, with an internal diameter of 0.8–1.2 mm, and with walls 0.2–0.3 mm thick.

The thermometer is preferably one that conforms to the specifications provided under Thermometers, Appendix I, selected for the desired accuracy and range of temperature.

PROCEDURE FOR CLASS I

Reduce the sample to a very fine powder, and unless otherwise directed, render it anhydrous when it contains water of hydration by drying it at the temperature specified in the monograph, or when the substance contains no water of hydration, dry it over a suitable desiccant for 16–24 h.

Charge a capillary glass tube, one end of which is sealed, with a sufficient amount of the dry powder to form a column in the bottom of the tube 2.5–3.5 mm high when packed down as closely as possible by moderate tapping on a solid surface.

Heat the bath until a temperature approximately 30° below the expected melting point is reached, attach the capillary tube to the thermometer, and adjust its height so that the material in the capillary is level with the thermometer bulb. Return the thermometer to the bath, continue the heating, with constant stirring, at a rate of rise of approximately 3°/min until a temperature 3° below the expected melting point is attained, then carefully regulate the rate to about 1°–2°/min until melting is complete.

The temperature at which the column of the sample is observed to collapse definitely against the side of the tube at any point is defined as the beginning of melting, and the temperature at which the sample becomes liquid throughout is defined as the end of melting. The two temperatures fall within the limits of the melting range.

PROCEDURE FOR CLASS IA

Prepare the sample and charge the capillary glass tube as directed for Class I. Heat the bath until a temperature 10 ± 1° below the expected melting range is reached, then introduce the charged tube, and heat at a rate of rise of 3 ± 0.5°/min until melting is complete. Record the melting range as for Class I.

PROCEDURE FOR CLASS IB

Place the sample in a closed container, and cool to 10° or lower for at least 2 h. Without previous powdering, charge the cooled material into the capillary tube as directed for Class I, immediately place the charged tube in a vacuum desiccator, and dry at a pressure not exceeding 20 mm Hg for 3 h. Immediately upon removal from the desiccator, fire-seal the open end of the tube. As soon as is practicable, proceed with the determination of the melting range as follows: Heat the bath until a temperature of 10 ± 1° below the expected melting range is reached, then introduce the charged tube, and heat at a rate of rise of 3 ± 0.5°/min until melting is complete. Record the melting range as directed in Class I.

If the particle size of the material is too large for the capillary, precool the sample as directed above, then with as little pressure as possible, gently crush the particles to fit the capillary, and immediately charge the tube.

PROCEDURE FOR CLASS II

Carefully melt the material to be tested at as low a temperature as possible, and draw it into a capillary tube that is left open at both ends to a depth of about 10 mm. Cool the charged tube at 10°, or lower, for 24 h, or in contact with ice for at least 2 h. Then attach the tube to the thermometer by means of a rubber band, adjust it in a water bath so that the upper edge of the material is 10 mm below the water level, and heat as directed for Class I, except within 5° of the expected melting temperature, regulate the rate of rise of temperature to 0.5°–1.0°/min. The temperature at which the material is observed to rise in the capillary tube is the melting temperature.

PROCEDURE FOR CLASS III

Melt a quantity of the substance slowly, while stirring, until it reaches a temperature of 90°–92°. Remove the source of heat, and allow the molten substance to cool to a temperature of 8°–10° above the expected melting point. Chill the bulb of an ASTM 14C thermometer (see Appendix I) to 5°, wipe it dry, and while it is still cold, dip it into the molten substance so that approximately the lower half of the bulb is submerged. Withdraw it immediately, and hold it vertically away from the heat until the wax surface dulls, then dip it for 5 min into a water bath having a temperature not higher than 16°.

Fix the thermometer securely in a test tube so that the lower point is 15 mm above the bottom of the test tube. Suspend the test tube in a water bath adjusted to about 16°, and raise the temperature of the bath at the rate of 2°/min to 30°, then change to a rate of 1°/min, and note the temperature at which the first drop of melted substance leaves the thermometer.

Repeat the determination twice on a freshly melted portion of the sample. If the variation of three determinations is less than 1°, take the average of the three as the melting point. If the variation of three determinations is greater than 1°, make two additional determinations and take the average of the five.
Optical (Specific) Rotation

Many chemicals in a pure state or in solution are optically active in the sense that they cause incident polarized light to emerge in a plane forming a measurable angle with the plane of the incident light. When this effect is large enough for precise measurement, it may serve as the basis for an assay or an identity test. In this connection, the optical rotation is expressed in degrees, as either angular rotation (observed) or specific rotation (calculated with reference to the specific concentration of 1 g of solute in 1 mL of solution, measured under stated conditions).

Specific rotation of a liquid substance usually is expressed:

\[ [\alpha]_s = \frac{a}{l} \]

For solutions of solid substances, is expressed:

\[ [\alpha]_s = \frac{100a}{ld} = \frac{100a}{lc} \]

t = temperature
x = wavelength of the light used
a = corrected observed rotation (°)
l = length of the polarimeter cell (dm)
d = specific gravity of the liquid or solution at the temperature of observation
p = concentration of the solution (number of grams of substance in 100 g of solution)
c = concentration of the solution (number of grams of substance in 100 mL of solution)

The concentrations p and c should be calculated on the dried or anhydrous basis, unless otherwise specified. Spectral lines most frequently employed are the D line of sodium (doublet at 589.0 nm and 589.6 nm) and the yellow-green line of mercury at 546.1 nm. The specific gravity and the rotatory power vary appreciably with the temperature.

The accuracy and precision of optical rotatory measurements will be increased if they are carried out with due regard for the following general considerations.

Supplement the source of illumination with a filtering system capable of transmitting light of a sufficiently monochromatic nature. Precision polarimeters generally are designed to accommodate interchangeable disks to isolate the D line from sodium light or the 546.1-nm line from the mercury spectrum. With polarimeters not thus designed, cells containing suitably colored liquids may be employed as filters.¹

Pay special attention to temperature control of the solution and of the polarimeter. Make accurate and reproducible observations to the extent that differences between replicates, or between observed and true values of rotation (the latter value having been established by calibration of the polarimeter scale with suitable standards), calculated in terms of either specific rotation or angular rotation, whichever is appropriate, do not exceed one-fourth of the range given in the individual monograph for the rotation of the article being tested. Generally, a polarimeter accurate to 0.05° of angular rotation, and capable of being read with the same precision, suffices for FCC purposes; in some cases, a polarimeter accurate to 0.01°, or less, of angular rotation, and read with comparable precision, may be required.

Fill polarimeter tubes in such a way as to avoid creating or leaving air bubbles, which interfere with the passage of the beam of light. Interference from bubbles is minimized with tubes in which the bore is expanded at one end. However, tubes of uniform bore, such as semimicro- or micro-tubes, require care for proper filling. At the time of filling, the tubes and the liquid or solution should be at a temperature not higher than that specified for the determination to guard against the formation of a bubble upon cooling and contraction of the contents.

In closing tubes having removable end plates fitted with gaskets and caps, the latter should be tightened only enough to ensure a leak-proof seal between the end plate and the body of the tube. Excessive pressure on the end plate may set up strains that result in interference with the measurements. In determining the specific rotation of a substance of low rotatory power, loosen the caps and tighten them again between successive readings in the measurement of both the rotation and the zero point. Differences arising from end plate strain thus generally will be revealed and appropriate adjustments to eliminate the cause may be made.

PROCEDURE

In the case of a solid, dissolve the substance in a suitable solvent, reserving a separate portion of the latter for a blank determination. Make at least five readings of the rotation of the solution, or of the substance itself if liquid, at 25° or the temperature specified in the individual monograph. Replace the solution with the reserved portion of the solvent (or, in the case of a liquid, use the empty tube), make the same number of readings, and use the average as the zero point value. Subtract the zero point value from the average observed rotation if the two figures are of the same sign, or add if opposite in sign, to obtain the corrected observed rotation.

[NOTE—Where a photoelectric polarimeter is used, a single measurement, corrected for the solvent blank, is made.]

CALCULATION

Calculate the specific rotation of a liquid substance, or of a solid in solution, by application of one of the following formulas:

1. for liquid substances:

\[ \left[ \alpha \right]_x = \frac{a}{ld} \]

2. for solutions of solids:

\[ \left[ \alpha \right]_x = \frac{100a}{pd} = \frac{100a}{lc} \]

\( t \) = temperature

\( x \) = wavelength of the light used

\( a \) = corrected observed rotation (°)

\( l \) = length of the polarimeter cell (dm)

\( d \) = specific gravity of the liquid or solution at the temperature of observation

\( p \) = concentration of the solution (number of grams of substance in 100 g of solution)

\( c \) = concentration of the solution (number of grams of substance in 100 mL of solution)

The concentrations \( p \) and \( c \) should be calculated on the dried or anhydrous basis, unless otherwise specified.

**pH Determination**

**PRINCIPLE**

The definition of pH is the negative log of the hydrogen ion concentration in moles per liter of aqueous solutions. Measure pH potentiometrically by using a pH meter or colorimetrically by using pH indicator paper.

**SCOPE**

This method is suitable to determine the pH of aqueous solutions. While pH meters, calibrated with aqueous solutions, are sometimes used to make measurements in semiaqueous solutions or in nonaqueous polar solutions, the value obtained is the apparent pH value only and should not be compared with the pH of aqueous solutions. For nonpolar solutions, pH has no meaning, and pH electrodes may be damaged by direct contact with these solutions. References to the pH of nonpolar solutions or liquids usually indicate the pH of a water extract of the nonpolar liquid or the apparent pH of a mixture of the nonpolar liquid in a polar liquid such as alcohol or alcohol–water mixtures.

**PROCEDURE** [POTENTIOMETRIC METHOD (pH METER)]

**Calibration:** Select two standard buffers to bracket, if possible, the anticipated pH of the unknown substances. These commercially available standards and the sample should be at the same temperature, within 2°. Set the temperature compensator of the pH meter to the temperature of the samples and standards. Follow the manufacturer’s instructions for setting temperature compensation and for adjusting the output during calibration. Rinse the electrodes with distilled or deionized water, and blot them dry with clean, absorbent laboratory tissue. Place the electrode(s) in the first standard buffer solution, and adjust the standardization control so that the pH reading matches the stated pH of the standard buffer. Repeat this procedure with fresh portions of the first buffer solution until two successive readings are within ±0.02 pH units with no further adjustment. Rinse the electrodes, blot them dry, and place them in a portion of the second standard buffer solution. Following the manufacturer’s instructions, adjust the slope control (not the standardization control) until the output displays the pH of the second standard buffer.

Repeat the sequence of standardization with both buffers until pH readings are within ± 0.02 pH units for both buffers without adjustments to either the slope or standardization controls. The pH of the unknown may then be measured, using either a pH electrode in combination with a reference electrode or a single combination electrode. Select electrodes made of chemically resistant glass when measuring samples of either low or high pH.

**pH Indicator paper:** Test papers impregnated with acid–base indicators, although less accurate than pH meters, offer a convenient way to determine the pH of an aqueous solution. They may be purchased in rolls or strips covering all or part of the pH range; papers covering a narrow part of the pH range can be sensitive to differences of 0.2 pH units. Some test papers comprise a plastic strip with small squares of test paper attached. The different squares are sensitive to different pH ranges. When using this type of test paper, wet all of the squares with the test sample to ensure a correct pH reading.

Test paper can contaminate the sample being tested; therefore, do not dip it into the sample. Either use a clean glass rod to remove a drop of the test solution and place it on the test paper, or transfer a small amount of the sample to a small container, dip the test paper into this portion, and compare the developed color with the color comparison chart provided with the test paper to determine the pH of the sample.
Sulfuric acid, 95%: Add a quantity of sulfuric acid of known concentration to sufficient water to adjust the final concentration to 94.5%–95.5% of H₂SO₄. Because the acid concentration may change upon standing or upon intermittent use, check the concentration frequently and either adjust solutions assaying more than 95.5% or less than 94.5% by adding either diluted or fuming sulfuric acid, as required, or discard them.

Cobaltous chloride CS: Dissolve about 65 g of cobaltous chloride (CoCl₂·6H₂O) in enough of a mixture of 25 mL of hydrochloric acid and 975 mL of water to make 1000 mL. Pipet 5 mL of this solution into a 250-mL iodine flask, add 5 mL of hydrogen peroxide TS (3%) and 15 mL of a solution of sodium hydroxide (1:5), boil for 10 min, cool, and add 2 g of potassium iodide and 20 mL of sulfuric acid (1:4). When the precipitate has dissolved, titrate the liberated iodine with 0.1 N sodium thiosulfate. The titration is sensitive to air oxidation and should be blanketed with carbon dioxide. Each mL of 0.1 N sodium thiosulfate is equivalent to 23.79 mg of CoCl₂·6H₂O. Adjust the final volume of the solution by adding enough of the mixture of hydrochloric acid and water so that each mL contains 59.5 mg of CoCl₂·6H₂O.

Cupric sulfate CS: Dissolve about 65 g of cupric sulfate (CuSO₄·5H₂O) in enough of a mixture of 25 mL of hydrochloric acid and 975 mL of water to make 1000 mL. Pipet 10 mL of this solution into a 250-mL iodine flask; add 40 mL of water, 4 mL of acetic acid, and 3 g of potassium iodide; and titrate the liberated iodine with 0.1 N sodium thiosulfate, adding starch TS as the indicator. Each mL of 0.1 N sodium thiosulfate is equivalent to 24.97 mg of CuSO₄·5H₂O. Adjust the final volume of the solution by adding enough of the mixture of hydrochloric acid and water so that each mL contains 62.4 mg of CuSO₄·5H₂O.

Ferric chloride CS: Dissolve about 55 g of ferric chloride (FeCl₃·6H₂O) in enough of a mixture of 25 mL of hydrochloric acid and 975 mL of water to make 1000 mL. Pipet 10 mL of this solution into a 250-mL iodine flask; add 15 mL of water, 5 mL of hydrochloric acid, and 3 g of potassium iodide; and allow the mixture to stand for 15 min. Dilute with 100 mL of water, and titrate the liberated iodine with 0.1 N sodium thiosulfate, adding starch TS as the indicator. Perform a blank determination with the same quantities of the same reagents and in the same manner, and make any necessary correction. Each mL of 0.1 N sodium thiosulfate is equivalent to 27.03 mg of FeCl₃·6H₂O. Adjust the final volume of the solution by adding the mixture of hydrochloric acid and water so that each mL contains 45.0 mg of FeCl₃·6H₂O.

Platinum–cobalt CS: Transfer 1.246 g of potassium chloroplatinate (K₂PtCl₆) and 1.00 g of crystallized cobaltous chloride (CoCl₂·6H₂O) into a 1000-mL volumetric flask, dissolve in about 200 mL of water and 100 mL of hydrochloric acid, dilute with water to volume, and mix. This solution has a color of 500 APHA units. [NOTE—Use this solution only when specified in an individual monograph.]

Matching Fluids

For purposes of comparison, a series of 20 matching fluids, each designated by a letter of the alphabet, is provided, the composition of each being as indicated in the accompanying table. To prepare the matching fluid specified, pipet the prescribed volumes of the colorimetric test solutions (CS) and water into one of the matching containers, and mix the solutions in the container.

<table>
<thead>
<tr>
<th>Matching Fluid</th>
<th>Parts of Cobaltous Chloride CS</th>
<th>Parts of Ferric Chloride CS</th>
<th>Parts of Cupric Sulfate CS</th>
<th>Parts of Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.1</td>
<td>0.4</td>
<td>0.1</td>
<td>4.4</td>
</tr>
<tr>
<td>B</td>
<td>0.3</td>
<td>0.9</td>
<td>0.3</td>
<td>8.5</td>
</tr>
<tr>
<td>C</td>
<td>0.1</td>
<td>0.6</td>
<td>0.1</td>
<td>4.2</td>
</tr>
<tr>
<td>D</td>
<td>0.3</td>
<td>0.6</td>
<td>0.4</td>
<td>3.7</td>
</tr>
<tr>
<td>E</td>
<td>0.4</td>
<td>1.2</td>
<td>0.3</td>
<td>3.1</td>
</tr>
<tr>
<td>F</td>
<td>0.3</td>
<td>1.2</td>
<td>0.0</td>
<td>3.5</td>
</tr>
<tr>
<td>G</td>
<td>0.5</td>
<td>1.2</td>
<td>0.2</td>
<td>3.1</td>
</tr>
</tbody>
</table>

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Matching Fluids* (continued)

<table>
<thead>
<tr>
<th>Matching Fluid</th>
<th>Parts of Cobaltous Chloride CS</th>
<th>Parts of Ferric Chloride CS</th>
<th>Parts of Cupric Sulfate CS</th>
<th>Parts of Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>H</td>
<td>0.2</td>
<td>1.5</td>
<td>0.0</td>
<td>3.3</td>
</tr>
<tr>
<td>I</td>
<td>0.4</td>
<td>2.2</td>
<td>0.1</td>
<td>2.3</td>
</tr>
<tr>
<td>J</td>
<td>0.4</td>
<td>3.5</td>
<td>0.1</td>
<td>1.0</td>
</tr>
<tr>
<td>K</td>
<td>0.5</td>
<td>4.5</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>L</td>
<td>0.8</td>
<td>3.8</td>
<td>0.1</td>
<td>0.3</td>
</tr>
<tr>
<td>M</td>
<td>0.1</td>
<td>2.0</td>
<td>0.1</td>
<td>2.8</td>
</tr>
<tr>
<td>N</td>
<td>0.0</td>
<td>4.9</td>
<td>0.1</td>
<td>0.0</td>
</tr>
<tr>
<td>O</td>
<td>0.1</td>
<td>4.9</td>
<td>0.1</td>
<td>0.0</td>
</tr>
<tr>
<td>P</td>
<td>0.2</td>
<td>0.4</td>
<td>0.1</td>
<td>4.3</td>
</tr>
<tr>
<td>Q</td>
<td>0.2</td>
<td>0.3</td>
<td>0.1</td>
<td>4.4</td>
</tr>
<tr>
<td>R</td>
<td>0.3</td>
<td>0.4</td>
<td>0.2</td>
<td>4.1</td>
</tr>
<tr>
<td>S</td>
<td>0.2</td>
<td>0.1</td>
<td>0.0</td>
<td>4.7</td>
</tr>
<tr>
<td>T</td>
<td>0.5</td>
<td>0.5</td>
<td>0.4</td>
<td>3.6</td>
</tr>
</tbody>
</table>


Refractive Index

The refractive index of a transparent substance is the ratio of the velocity of light in air to its velocity in that material under like conditions. It is equal to the ratio of the sine of the angle of incidence made by a ray in air to the sine of the angle of refraction made by the ray in the material being tested. The refractive index values specified in this Codex are for the D line of sodium (589 nm) unless otherwise specified. The determination should be made at the temperature specified in the individual monograph, or at 25° if no temperature is specified. This physical constant is used as a means for identification of, and detection of impurities in, volatile oils and other liquid substances. The Abbé refractometer, or other refractometers of equal or greater accuracy, may be employed at the discretion of the operator.

Solidification Point

SCOPE

This method is designed to determine the solidification point of food-grade chemicals having appreciable heats of fusion. It is applicable to chemicals having solidification points between −20° and +150°. Necessary modifications will be noted in individual monographs.

DEFINITION

Solidification Point is an empirical constant defined as the temperature at which the liquid phase of a substance is in approximate equilibrium with a relatively small portion of the solid phase. It is measured by noting the maximum temperature reached during a controlled cooling cycle after the appearance of a solid phase.

The solidification point is distinguished from the freezing point in that the latter term applies to the temperature of equilibrium between the solid and liquid state of pure compounds.

Some chemical compounds have more than one temperature at which there may be an equilibrium between the solid and liquid state depending on the crystal form of the solid that is present.

APPARATUS

The apparatus illustrated in Figures 3 and 4 consists of the components described in the following paragraphs.
Thermometer: A thermometer having a range not exceeding 30°, graduated in 0.1° divisions, and calibrated for 76-mm immersion should be employed. A satisfactory series of thermometers, covering a range from −20° to +150°, is available as ASTM-E1 89C through 96C (see Thermometers, Appendix I). A thermometer should be chosen such that the solidification point is not obscured by the cork stopper of the sample container.

Sample container: Use a standard glass 25-mm × 150-mm test tube with a lip, fitted with a two-hole cork stopper to hold the thermometer in place and to allow adequate stirring with a stirrer.

Air jacket: For the air jacket, use a standard glass 38-mm × 200-mm test tube with a lip and fitted with a cork or rubber stopper bored with a hole into which the sample container can easily be inserted up to the lip.

Cooling bath: Use a 2000-mL beaker or a similar, suitable container as a cooling bath. Fill it with an appropriate cooling medium such as glycerin, mineral oil, water, water and ice, or alcohol–dry ice.

Stirrer: The stirrer (Figure 4) consists of a 1-mm in diameter (B & S gauge 18), corrosion-resistant wire bent into a series of three loops about 25 mm apart. It should be made so that it will move freely in the space between the thermometer and the inner wall of the sample container. The shaft of the stirrer should be of a convenient length designed to pass loosely through a hole in the cork holding the thermometer. Stirring may be hand operated or mechanically activated at 20–30 strokes/min.

Assembly: Assemble the apparatus in such a way that the cooling bath can be heated or cooled to control the desired temperature ranges. Clamp the air jacket so that it is held rigidly just below the lip, and immerse it in the cooling bath to a depth of 160 mm.

SAMPLE PREPARATION

The solidification point of chemicals is usually determined as they are received. Some may be hygroscopic, however, and will require special drying. If this is necessary, it will be noted in the individual monographs.
Viscosity Determination

Viscosity is a fluid’s measured internal resistance to flow. Thick, slow-moving fluids have higher viscosities than thin, free-flowing fluids. The basic unit of measure for viscosity is the poise or Pascal second, Pa · s, in SI units. The relationship between poise and Pa · s is 1 poise = 0.1 Pa · s. Since commonly encountered viscosities are often fractions of 1 poise, viscosities are commonly expressed as centipoises (one centipoise = 0.01 poise). Poise or centipoise is the unit of measure for absolute viscosity. Kinematic viscosity also is commonly used and is determined by dividing the absolute viscosity of the test liquid by the density of the test liquid at the same temperature as the viscosity measurement and is expressed as stokes or centistokes (poise/density = stokes). The specified temperature is important: viscosity varies greatly with temperature, generally decreasing with increasing temperature.

Absolute viscosity can be determined directly if accurate dimensions of the measuring instruments are known. It is common practice to calibrate an instrument with a fluid of known viscosity and to determine the unknown viscosity of another fluid by comparison with that of the known viscosity.

Many substances, such as gums, have a variable viscosity, and most of them are less resistant to flow at higher flow (more correctly, shear) rates. In such cases, select a given set of conditions for measurement, and consider the measurement obtained to be an apparent viscosity. Since a change in the conditions of measurement would yield a different value for the apparent viscosity of such substances, the operator must closely adhere to the instrument dimensions and conditions for measurement.

MEASURING VISCOITY

Several common methods are available for measuring viscosity. Two very common ones are the use of capillary tubes such as Ubbelohde, Ostwald, or Cannon-Fenske viscometer tubes and the use of a rotating spindle such as the Brookfield viscometer.

Determine the viscosity in capillary tubes by measuring the amount of time it takes for a given volume of liquid to flow through a calibrated capillary tube. Calibrate the capillary tube by using liquids of known viscosity. The calibration may be obtained to be an apparent viscosity. Since a change in the conditions of measurement would yield a different value for the apparent viscosity of such substances, the operator must closely adhere to the instrument dimensions and conditions for measurement.

\[
k = \frac{v}{dt}
\]

in which \( v \) is the known viscosity, in centipoises, of the standard liquid; \( d \) is the density, at the specified temperature, of the liquid; and \( t \) is the time, in seconds, for the liquid to pass from the upper mark to the lower mark. It is not necessary to recalibrate the tube unless changes or repairs are made to it. To measure viscosity, introduce the unknown liquid into the
viscometer tube in the same way as the calibration standard was introduced, and measure the time, in seconds, it takes for
the liquid to flow from the upper mark to the lower mark. Calculate viscosity:

\[ v = kdt \]

in which \( v \) is the viscosity to be determined, \( k \) is the viscometer constant, and \( d \) is the density of the liquid being measured.

Using rotational viscometers provides a particularly rapid and convenient method for determining viscosity. They
employ a rotating spindle or cup immersed in the liquid, and they measure the resistance of the liquid to the rotation of the
spindle or cup. A wide range of viscosities can be measured with one instrument by using spindles or cups of different sizes
and by rotating them at different speeds. The manufacturer supplies the calibration of viscosity versus the spindle size and
speed, which can be checked by using fluids of known viscosity. Take a measurement by allowing the sample to come to the
desired temperature in a constant-temperature bath and immersing the spindle or cup to the depth specified by the
manufacturer. Allow the spindle or cup to rotate until a constant reading is obtained. Multiply the reading by a factor
supplied by the manufacturer for a given spindle or cup and given rotational speed to obtain the viscosity. The exact
procedures will vary with the particular instrument. An example is given in the section on Viscosity of Cellulose Gum.

Another method to determine viscosity uses the falling-ball viscometer. Determine viscosity by noting the time it takes
for a ball to fall through the distance between two marks on a tube filled with the unknown liquid (the tube is generally in a
constant-temperature bath). Use balls of different weights to measure a wide range of viscosities. Calculate the viscosity by
using manufacturer-supplied constants for the ball used. These instruments can be quite precise for Newtonian liquids, that
is, liquids that do not have viscosities that vary with flow (more correctly, shear) rate.

Three specific methods are described below:

**VISCOITY OF DIMETHYLPOLYSILOXANE**

**Apparatus:** The Ubbelohde suspended level viscometer, shown in Figure 5 is preferred to determine the viscosity of
dimethylpolysiloxane. Alternatively, a Cannon-Ubbelohde viscometer may be used.

![Ubbelohde Viscometer for Dimethylpolysiloxane](image)

Select a viscometer having a minimum flow time of at least 200 s. Use a No. 3 size Ubbelohde, or a No. 400 size Cannon-
Ubbelohde, viscometer for the range of 300–600 centistokes. The viscometer should be fitted with holders that satisfy the
dimensional positions of the separate tubes as shown in the diagram and that hold the viscometer vertically. Filling lines in
bulb A indicate the minimum and maximum volumes of liquid to be used for convenient operation. The volume of bulb B is
approximately 5 mL.

**Calibration of the viscometer:** Determine the viscosity constant, \( C \), for each viscometer by using an oil of known viscosity.\(^2\)
Charge the viscometer by tilting the instrument about 30 degrees from the vertical, with bulb A below the capillary, and
then introduce enough of the sample into tube I to bring the level up to the lower filling line. The level should not be above
the upper filling line when the viscometer is returned to the vertical position and the sample has drained from tube I. Charge
the viscometer in such a manner that the U-tube at the bottom fills completely without trapping air.

After the viscometer has been in a constant-temperature bath (25 ± 0.2°) long enough for the sample to reach
temperature equilibrium, place a finger over tube 3, and apply suction to tube 2 until the liquid reaches the center of bulb C.
Remove suction from tube 2, then remove the finger from tube 3, and place it over tube 2 until the sample drops away from

\(^2\)Oils of known viscosities may be obtained from the Cannon Instrument Co., P.O. Box 812, State College, PA 16801. For determining the viscosity of
dimethylpolysiloxane, choose an oil with a viscosity as close as possible to that of the type of sample to be tested.
the lower end of the capillary. Remove the finger from tube 2, and measure the time, to the nearest 0.1 s, required for the meniscus to pass from the first timing mark ($T_1$) to the second ($T_2$).

Calculate the viscometer constant, $C$:

$$C = \frac{cs}{t_1}$$

in which $cs$ is the viscosity, in centistokes, and $t_1$ is the efflux time, in seconds, for the standard liquid.

**Determination of the viscosity of dimethylpolysiloxane:** Charge the viscometer with the sample in the same manner as described for the calibration procedure; determine the efflux time, $t_2$; and calculate the viscosity of the dimethylpolysiloxane:

$$V = C \times t_2$$

**VISCOSITY OF METHYLCYLCELLULOSE**

**Apparatus:** Viscometers used to determine the viscosity of methylcellulose and some related compounds are illustrated in Figure 6 and consist of three parts: a large filling tube, A; an orifice tube, B; and an air vent to the reservoir, C.

![Figure 6. Methylcellulose Viscometers](image)

There are two basic types of methylcellulose viscometers—one for cellulose derivatives of a range between 1500 and 4000 centipoises, and the other for less viscous ones. Each type of viscometer is modified slightly for the different viscosities.

**Calibration of the viscometer:** Determine the viscometer constant, $K$, for each viscometer by using an oil of known viscosity. Place an excess of the liquid that is to be tested (adjusted to 20 ± 0.1°) in the filling tube, A, and transfer it to the orifice tube, B, by gentle suction, taking care to keep the liquid free from air bubbles by closing the air vent tube, C. Adjust the column of liquid in tube B so it is even with the top graduation line. Open both tubes B and C to permit the liquid to flow into the reservoir against atmospheric pressure.

**NOTE—**Failure to open air vent tube C before determining the viscosity will yield false values.

Record the time, in seconds, for the liquid to flow from the upper mark to the lower mark in tube B.

Calculate the viscometer constant, $K$:

$$K = \frac{V}{d \times t}$$

in which $V$ is the viscosity, in centipoises, of the liquid; $K$ is the viscometer constant; $d$ is the specific gravity of the liquid tested at 20°/20°; and $t$ is the time, in seconds, for the liquid to pass from the upper to the lower mark.

For the calibration, all values in the equation are known or can be determined except $K$, which must be solved. If a tube is repaired, it must be recalibrated to avoid obtaining significant changes in the value of $K$.

**Determination of the viscosity of methylcellulose:** Prepare a 2% solution of methylcellulose or other cellulose derivative, by weight, as directed in the monograph. Place the solution in the proper viscometer and determine the time, $t$, required for
the solution to flow from the upper mark to the lower mark in orifice tube B. Separately determine the specific gravity, d, at 20°/20°. Viscosity, V = Kdt.

VISCOSITY OF CELLULOSE GUM

Apparatus: Use a Brookfield Model LV series viscometer, analog or digital, or equivalent type viscometer for the determination of viscosity of aqueous solutions of cellulose gum within the range of 25–10,000 centipoises at 25°. Rotational viscometers of this type have spindles for use in determining the viscosity of different viscosity types of cellulose gum. The spindles and speeds for determining viscosity within different ranges are tabulated below.

<table>
<thead>
<tr>
<th>Viscosity Range (centipoises)</th>
<th>Spindle No.</th>
<th>Speed (rpm)</th>
<th>Scale</th>
<th>Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>10–100</td>
<td>1</td>
<td>60</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>100–200</td>
<td>1</td>
<td>30</td>
<td>100</td>
<td>2</td>
</tr>
<tr>
<td>200–1000</td>
<td>2</td>
<td>30</td>
<td>100</td>
<td>10</td>
</tr>
<tr>
<td>1000–4000</td>
<td>3</td>
<td>30</td>
<td>100</td>
<td>40</td>
</tr>
<tr>
<td>4000–10,000</td>
<td>4</td>
<td>30</td>
<td>100</td>
<td>200</td>
</tr>
</tbody>
</table>

Mechanical stirrer: Use an agitator, essentially as shown in Figure 7, that can be attached to a variable-speed motor capable of operating at 900 ± 100 rpm under varying load conditions.

Figure 7. Agitator for Viscosity of Cellulose Gum

[NOTE—The agitator may be fabricated from stainless steel (Hercules, Inc., Wilmington, Delaware, or equivalent.) or glass as shown in Figure 7. Where this procedure is specified for viscosity measurements by reference in other monographs, equivalent three-blade agitators may be used.]

Sample container: Use a glass jar about 152 mm deep having an od of approximately 64 mm and a capacity of about 340 g.

Water bath: Use a water bath capable of maintaining a constant temperature. Set the temperature to 25°, and maintain it within ±0.2°.

Thermometer: Use an ASTM Saybolt Viscosity Thermometer having a range from 19° to 27° and conforming to the requirements for Thermometer 17C as described in ASTM Specification E1.

Sample preparation: Accurately weigh an amount of sample equivalent to 4.8 g of cellulose gum on the dried basis, and record the actual quantity required, in grams, as S. Transfer an accurately measured volume of water equivalent to 240 – S g into the sample container. Position the stirrer in the sample container, allowing minimal clearance between the stirrer and the bottom of the container. Begin stirring, and slowly add the sample. Adjust the stirring speed to approximately 900 ± 100 rpm. Mix for exactly 2 h. Do not allow the stirring speed to exceed 1200 rpm. Remove the stirrer, cap the sample.
container, and transfer the sample container into a constant-temperature water bath, maintained at 25 ± 0.2°, for 1 h. Check the sample temperature with a thermometer at the end of 1 h to ensure that the test temperature has been reached.

Procedure: Remove the sample container from the water bath, shake vigorously for 10 s, and measure the viscosity with the Brookfield viscometer, using the proper spindle and speed indicated in the accompanying table. Be sure to use the viscometer guard, and allow the spindle to rotate for 3 min before taking the reading. Calculate the viscosity, in centipoises, by multiplying the reading observed by the appropriate factor from the table.

**Water Determination**

**METHOD I (KARL FISCHER TITRIMETRIC METHOD)**

Determine the water by Method Ia, unless otherwise specified in the individual monograph.

**METHOD Ia (DIRECT TITRATION)**

**Principle:** The titrimetric determination of water is based on the quantitative reaction of water with an anhydrous solution of sulfur dioxide and iodine in the presence of a buffer that reacts with hydrogen ions.

In the original titrimetric solution, known as Karl Fischer Reagent, the sulfur dioxide and iodine are dissolved in pyridine and methanol. Pyridine-free reagents are more commonly used now. The test specimen may be titrated with the Karl Fischer Reagent directly, or the analysis may be carried out by a residual titration procedure. The stoichiometry of the reaction is not exact, and the reproducibility of the determination depends on such factors as the relative concentrations of the Karl Fischer Reagent ingredients, the nature of the inert solvent used to dissolve the test specimen, the apparent pH of the final mixture, and the technique used in the particular determination. Therefore, an empirically standardized technique is used to achieve the desired accuracy. Precision in the method is governed largely by the extent to which atmospheric moisture is excluded from the system. The titration of water is usually carried out with the use of anhydrous methanol as the solvent for the test specimen; however, other suitable solvents may be used for special or unusual test specimens.

Substances that may interfere with the test results are ferric ion, chlorine, and similar oxidizing agents, as well as significant amounts of strong acids or bases, phosgene, or anything that will reduce iodide to iodine, poison the reagent, and show the sample to be bone dry when water may be present (false negative). 8-Hydroxyquinoline may be added to the vessel to eliminate interference from ferric ion. Chlorine interference can be eliminated with sulfur dioxide or unsaturated hydrocarbon. Excess pyridine or other amines may be added to the vessel to eliminate the interference of strong acids. Excess acetic acid or other carboxylic acid can be added to reduce the interference of strong bases. Aldehydes and ketones may react with the solution, showing the sample to be wet while the detector never reaches an endpoint (false positive).

**Apparatus:** Any apparatus may be used that provides for adequate exclusion of atmospheric moisture and for determination of the endpoint. In the case of a colorless solution that is titrated directly, the endpoint may be observed visually as a change in color from canary yellow to amber. The reverse is observed in the case of a test specimen that is titrated residually. More commonly, however, the endpoint is determined electrometrically with an apparatus employing a simple electrical circuit that serves to impress about 200 mV of applied potential between a pair of platinum electrodes (about 5 mm² in area and about 2.5 cm apart) immersed in the solution to be titrated. At the endpoint of the titration, a slight excess of the reagent increases the flow of current to 50–150 microamperes for 30 s to 30 min, depending on the solution being titrated. The time is shortest for substances that dissolve in the reagent. The longer times are required for solid materials that do not readily go into solution in the Karl Fischer Reagent. With some automatic titrators, the abrupt change in current or potential at the endpoint serves to close a solenoid-operated valve that controls the buret delivering the titrant. A commercially available apparatus generally comprises a closed system consisting of one or two automatic burets and a tightly covered titration vessel fitted with the necessary electrodes and a magnetic stirrer. The air in the system is kept dry with a suitable desiccant such as phosphorus pentoxide, and the titration vessel may be purged by means of a stream of dry nitrogen or a current of dry air.

**Reagent:** The Karl Fischer Reagent may be prepared as follows: Add 125 g of iodine to a solution containing 670 mL of methanol and 170 mL of pyridine, and cool. Place 100 mL of pyridine in a 250-mL graduated cylinder, and keeping the pyridine cold in an ice bath, pass in dry sulfur dioxide until the volume reaches 200 mL. Slowly add this solution, with shaking, to the cooled iodine mixture. Shake to dissolve the iodine, transfer the solution to the apparatus, and allow the solution to stand overnight before standardizing. One mL of this solution, when freshly prepared, is equivalent to approximately 5 mg of water, but it deteriorates gradually; therefore, standardize it within 1 h before use, or daily in continual use. Protect the solution from light while in use. Store any bulk stock of the solution in a suitably sealed, glass-stoppered container, fully protected from light and under refrigeration.

A commercially available, stabilized solution of a Karl Fischer-type reagent may be used. Commercially available reagents containing solvents or bases other than pyridine and/or alcohols other than methanol also may be used. These may be single solutions or reagents formed in situ by combining the components of the reagents present in two discrete solutions. The diluted Karl Fischer Reagent called for in some monographs should be diluted as directed by the manufacturer. Either methanol, or another suitable solvent such as ethylene glycol monomethyl ether, may be used as the diluent.

**Test preparation:** Unless otherwise specified in the individual monograph, use an accurately weighed or measured amount of the specimen under test estimated to contain 10–250 mg of water.

Where the monograph specifies that the specimen under test is hygroscopic, accurately weigh a sample of the specimen into a suitable container. Use a dry syringe to inject an appropriate volume of methanol, or other suitable solvent, accurately measured, into the container and shake to dissolve the specimen. Dry the syringe, and use it to remove the solution from the container and transfer it to a titration vessel prepared as directed under Procedure. Repeat the procedure with a second portion of methanol, or other suitable solvent, accurately measured; add this washing to the titration vessel; and immediately titrate. Determine the water content, in milligrams, of a portion of solvent of the same total volume as that used to dissolve
the specimen and to wash the container and syringe, as directed under Standardization of water solution for residual titration, and subtract this value from the water content, in mg, obtained in the titration of the specimen under test.

**Standardization of the reagent:** Place enough methanol or other suitable solvent in the titration vessel to cover the electrodes, and add sufficient Karl Fischer Reagent to give the characteristic color or 100 ± 50 microamperes of direct current at about 200 mV of applied potential. Pure methanol can make the detector overly sensitive, particularly at low ppm levels of water, causing it to deflect to dryness and slowly recover with each addition of reagent. This slows down the titration and may allow the system to actually pick up ambient moisture during the resulting long titration. Adding chloroform or a similar nonconducting solvent will retard this sensitivity and can improve the analysis.

For determination of trace amounts of water (less than 1%), quickly add 25 µL (25 mg) of pure water, using a 25- or 50-µL syringe, and titrate to the endpoint. The water equivalence factor \( F \), in mg of water per mL of reagent, is given below:

\[
\text{Result} = 25/V
\]

in which \( V \) is the volume, in mL, of the Karl Fischer Reagent consumed in the second titration.

For the precise determination of significant amounts of water (more than 1%), quickly add 25–250 mg (25–250 µL) of pure water, accurately weighed by difference from a weighing pipet or from a precalibrated syringe or micropipet, the amount of water used being governed by the reagent strength and the buret size, as referred to under Volumetric Apparatus. Titrate to the endpoint. Calculate the water equivalence factor, \( F \), in mg of water per mL of reagent:

\[
\text{Result} = W/V
\]

in which \( W \) is the weight, in mg, of the water, and \( V \) is the volume, in mL, of the Karl Fischer Reagent required.

**Procedure:** Unless otherwise specified, transfer 35–40 mL of methanol or other suitable solvent to the titration vessel, and titrate with the Karl Fischer Reagent to the electrometric or visual endpoint to consume any moisture that may be present. (Disregard the volume consumed because it does not enter into the calculations.) Quickly add the Test Preparation, mix, and again titrate with the Karl Fischer Reagent to the electrometric or visual endpoint. Calculate the water content of the specimen, in mg:

\[
\text{Result} = SF
\]

in which \( S \) is the volume, in mL, of the Karl Fischer Reagent consumed in the second titration, and \( F \) is the water equivalence factor of the Karl Fischer Reagent.

**METHOD IB (RESIDUAL TITRATION)**

**Principle:** See the information in the section entitled Principle under Method Ia. In the residual titration, add excess Karl Fischer Reagent to the test specimen, allow sufficient time for the reaction to reach completion, and titrate the unconsumed Karl Fischer Reagent with a standard solution of water in a solvent such as methanol. The residual titration procedure is generally applicable and avoids the difficulties that may be encountered in the direct titration of substances from which the bound water is released slowly.

**Apparatus, Reagent, and Test preparation:** Use those in Method Ia.

**Standardization of water solution for residual titration:** Prepare a Water solution by diluting 2 mL of pure water to 1000 mL with methanol or another suitable solvent. Standardize this solution by titrating 25.0 mL with the Karl Fischer Reagent, previously standardized as directed under Standardization of the reagent. Calculate the water content, in mg/mL, of the Water solution:

\[
\text{Result} = VF/25
\]

in which \( V \) is the volume of the Karl Fischer Reagent consumed, and \( F \) is the water equivalence factor of the Karl Fischer Reagent. Determine the water content of the Water solution weekly, and standardize the Karl Fischer Reagent against it periodically as needed. Store the Water solution in a tightly capped container.

**Procedure:** Where the individual monograph specifies the water content is to be determined by Method Ib, transfer 35–40 mL of methanol or other suitable solvent into the titration vessel, and titrate with the Karl Fischer Reagent to the electrometric or visual endpoint. Quickly add the Test preparation, mix, and add an accurately measured excess of the Karl Fischer Reagent. Allow sufficient time for the reaction to reach completion, and titrate the unconsumed Karl Fischer Reagent with standardized Water solution to the electrometric or visual endpoint. Calculate the water content of the specimen, in mg:

\[
\text{Result} = F(X' - XR)
\]

in which \( F \) is the water equivalence factor of the Karl Fischer Reagent; \( X' \) is the volume, in mL, of the Karl Fischer Reagent added after introduction of the specimen; \( X \) is the volume, in mL, of standardized Water solution required to neutralize the unconsumed Karl Fischer Reagent; and \( R \) is the ratio \( V/25 \) (mL of Karl Fischer Reagent/mL of Water solution), determined from the Standardization of water solution for residual titration.

**METHOD IC (COULOMETRIC TITRATION)**

**Principle:** Use the Karl Fischer reaction in the coulometric determination of water. In this determination, iodine is not added in the form of a volumetric solution, but is produced in an iodide-containing solution by anodic oxidation. The reaction cell usually consists of a large anode compartment and a small cathode compartment that are separated by a diaphragm. Other suitable types of reaction cells (e.g., without diaphragms) may be used. Each compartment has a platinum electrode that
conducts current through the cell. Iodine, which is produced at the anode electrode, immediately reacts with the water present in the compartment. When all the water has been consumed, an excess of iodine occurs, which can be detected potentiometrically, thus indicating the endpoint. Pre-electrolysis, which can take several hours, eliminates moisture from the system. Therefore, changing the **Karl Fischer Reagent** after each determination is not practical. Individual determinations may be carried out in succession in the same reagent solution. A requirement for this method is that each component of the test specimen be compatible with the other components and that no side reactions take place. Samples may be transferred into the vessel as solids or as solutions by means of injection through a septum. Gases can be introduced into the cell by means of a suitable gas inlet tube. For the water determination of solids, another common technique is to dissolve the solid in a suitable solvent and then inject a portion of this solution into the cell. In the case of insoluble solids, water may be extracted using suitable solvents, and then the extracts injected into the coulometric cell. Alternatively, an evaporation technique may be used in which the sample is heated in a tube and the water is evaporated and carried into the cell by means of a stream of dry, inert gas. Precision in the method is predominantly governed by the extent to which atmospheric moisture is excluded from the system. Control of the system may be monitored by measuring the amount of baseline drift. The titration of water in solid test specimens is usually carried out with the use of anhydrous methanol as the solvent. Other suitable solvents may be used for special or unusual test specimens. This method is particularly suited to chemically inert substances such as hydrocarbons, alcohols, and ethers. In comparison with the volumetric Karl Fischer titration, coulometry is a micro-method. The method uses extremely small amounts of current. It is predominantly used for substances with a very low water content (0.1%–0.0001%).

**Apparatus:** Any commercially available apparatus consisting of an absolutely tight system fitted with the necessary electrodes and a magnetic stirrer is appropriate. The instrument’s microprocessor controls the analytical procedure and displays the results. Calibration of the instrument is not necessary as the current consumed can be measured absolutely. Proper operation of the instrument can be confirmed by injecting 1 μL of water into the vessel. The instrument should read 1000 μg of water on reaching the endpoint.

**Reagent:** See Reagent under Method Ia.

**Test preparation:** Using a dry syringe, inject an appropriate volume of test specimen estimated to contain 0.5–5 mg of water, accurately measured, into the anolyte solution. The sample may also be introduced as a solid, accurately weighed, into the anolyte solution. Perform coulometric titration, and determine the water content of the specimen under test.

Alternatively, when the specimen is a suitable solid, dissolve an appropriate quantity, accurately weighed, in anhydrous methanol or another suitable solvent, and inject a suitable portion into the anolyte solution. When the specimen is an insoluble solid, extract the water by using a suitable anhydrous solvent from which an appropriate quantity, accurately weighed, may be injected into the anolyte solution. Alternatively use an evaporation technique.

**Procedure:** Quickly inject the Test preparation, or transfer the solid sample, into the anolyte, mix, and perform the coulometric titration to the electrometric endpoint. Read the water content of the Test preparation directly from the instrument’s display, and calculate the percent that is present in the substance.

### METHOD II (TOLUENE DISTILLATION METHOD)

**Principle:** This method determines water by distillation of a sample with an immiscible solvent, usually toluene.

**Apparatus:** Use a glass distillation apparatus (see Figure 8) provided with 24/40 ground-glass connections. The components consist of a 500-mL short-neck, round-bottom flask connected by means of a trap to a 400-mm water-cooled condenser. The lower tip of the condenser should be about 7 mm above the surface of the liquid in the trap after distillation conditions have been established (see Procedure).
The trap should be constructed of well-annealed glass, the receiving end of which is graduated to contain 5 mL and subdivided into 0.1-mL divisions, with each 1-mL line numbered from 5 mL beginning at the top. Calibrate the receiver by adding 1 mL of water, accurately measured, to 100 mL of toluene contained in the distillation flask. Conduct the distillation, and calculate the volume of water obtained as directed in the Procedure. Add another mL of water to the cooled apparatus, and repeat the distillation. Continue in this manner until five 1-mL portions of water have been added. The error at any indicated capacity should not exceed 0.05 mL. The source of heat is either an oil bath or an electric heater provided with a suitable means of temperature control. The distillation may be better controlled by insulating the tube leading from the flask to the receiver. It is also advantageous to protect the flask from drafts. Clean the entire apparatus with potassium dichromate-sulfuric acid cleaning solution, rinse thoroughly, and dry completely before using.

Procedure: Place in the previously cleaned and dried flask a quantity of the substance, weighed accurately to the nearest 0.01 g, that is expected to yield 1.5–4 mL of water. If the substance is of a pastelike consistency, weigh it in a boat of metal foil that will pass through the neck of the flask. If the substance is likely to cause bumping, take suitable precautions to prevent it. Transfer about 200 mL of ACS reagent-grade toluene into the flask, and swirl to mix it with the sample. Assemble the apparatus, fill the receiver with toluene by pouring it through the condenser until it begins to overflow into the flask, and insert a loose cotton plug in the top of the condenser. Heat the flask so that the distillation rate will be about 200 drops/min, and continue distilling until the volume of water in the trap remains constant for 5 min. Discontinue the heating, use a copper or nichrome wire spiral to dislodge any drops of water that may be adhering to the inside of the condenser tube or receiver, and wash down with about 5 mL of toluene. Disconnect the receiver, immerse it in water at 25° for at least 15 min or until the toluene layer is clear, and then read the volume of water. Conduct a blank determination using the same volume of toluene as used when distilling the sample mixture, and make any necessary correction (see General Provisions).

C. OTHERS

Ash (Acid-Insoluble)

Boil the ash obtained as directed under Ash (Total), below, with 25 mL of 2.7 N hydrochloric acid for 5 min, collect the insoluble matter on a tared, porous-bottom porcelain filter crucible or ashless filter, wash it with hot water, ignite to constant weight at 675 ± 25°, and weigh. Calculate the percent acid-insoluble ash from the weight of the sample taken.

[NOTE—Avoid exposing the crucible to sudden temperature changes.]
Ash (Total)

Unless otherwise directed, accurately weigh about 3 g of the sample in a tared crucible, ignite it at a low temperature (about 550°), not to exceed a very dull redness, until it is free from carbon, cool it in a desiccator, and weigh. If a carbon-free ash is not obtained, wet the charred mass with hot water, collect the insoluble residue on an ashless filter paper, and ignite the residue and filter paper until the ash is white or nearly so. Finally, add the filtrate, evaporate it to dryness, and heat the whole to a dull redness. If a carbon-free ash is still not obtained, cool the crucible, add 15 mL of ethanol, break up the ash with a glass rod, then burn off the ethanol, again heat the whole to a dull redness, cool it in a desiccator, and weigh.

Hydrochloric Acid Table

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<td>36.93</td>
</tr>
<tr>
<td>23.0</td>
<td>1.1885</td>
<td>37.14</td>
</tr>
<tr>
<td>23.1</td>
<td>1.1895</td>
<td>37.36</td>
</tr>
<tr>
<td>23.2</td>
<td>1.1904</td>
<td>37.58</td>
</tr>
<tr>
<td>23.3</td>
<td>1.1914</td>
<td>37.80</td>
</tr>
<tr>
<td>23.4</td>
<td>1.1924</td>
<td>38.03</td>
</tr>
</tbody>
</table>
Specific gravity determinations were made at 60°F, compared with water at 60°F. From the specific gravities, the corresponding degrees Baumé were calculated by the following formula:

\[
\text{degrees Baumé} = 145 - \left(\frac{145}{\text{sp. gr.}}\right)
\]

Baumé hydrometers for use with this table must be graduated by the above formula, which should always be printed on the scale.

**ALLOWANCE FOR TEMPERATURE**

- 10°–15°Bé: 1/40 °Bé or 0.0002 sp. gr. for 1°F
- 15°–22°Bé: 1/30 °Bé or 0.0003 sp. gr. for 1°F
- 22°–25°Bé: 1/28 °Bé or 0.00035 sp. gr. for 1°F

**Insoluble Foreign Matter in Amino Acids**

This method was developed to detect insoluble foreign contaminants such as fibers, metal, glass, insects, hair, and other foreign matter that may be present in amino acid ingredients.

**REAGENTS**

**Diluent:** 2.5 N sodium hydroxide unless otherwise specified in the individual monograph. [Note—Filter before use (100 μm or less).]

**Apparatus:** Assemble a vacuum filter flask apparatus with a 100-μm nylon net filter as described in Figure 9.
Figure 9. Vacuum Filter Apparatus (1. Clamped funnel, diameter about 5 cm, and clamp; 2. suction flask attached to clamped funnel with a rubber stopper/sleeve with one hole; 3. 100-µm nylon net filter\(^4\) pre-dried and cooled (see Procedure); 4. vacuum line connection.)

**SAMPLING**

**Composite sample:** Combine and thoroughly mix the samples collected as prescribed below in the *Sampling plan*. Use a 100 g aliquot of this mixture as the test *Composite sample*, unless otherwise indicated in the monograph.

**Sampling plan:** For consignments comprising less than or equal to 3 containers, collect representative samples from each container in the consignment. For consignments comprising greater than or equal to 4 containers, take a representative sample from each of the \(n\) containers in the consignment where:

\[
\text{n} = 1.5 \times \text{square root}(N)
\]

\(N\) = total number of containers comprising the consignment

*[NOTE—Appropriate measures should be taken to ensure that samples withdrawn from containers are representative of the entire container, especially for containers much larger than 50 kg. The USP general chapter *Bulk Powder Sampling Procedures* <1097> is one guidance standard that may be useful for this purpose. Additionally, for consignments far exceeding 100 containers, other applicable Codex Alimentarius and ISO standards may be consulted for appropriate sampling procedures.]*

**Sample solution:** Quantitatively transfer the *Composite sample* into a glass beaker, add 500 mL *Diluent*, and stir until the sample is completely dissolved. *[NOTE—*Diluent* can be used to aid in the quantitative transfer of the *Composite sample* into the glass beaker. Do not use a magnetic stirrer as it may trap ferric and/or ferrous contaminants.]*

**PROCEDURE**

*[NOTE—Operation shall be done on a clean bench or a booth. To avoid contamination during the preparation, use clean utensils.]*

Pre-dry the 100-µm nylon net filter\(^4\) in a glass beaker at 90° for 1 h. Cool the dried nylon net filter in a desiccator for 1 h and weigh the initial nylon net filter to the nearest 0.1 mg \((X_1)\).

Filter the *Sample solution* through the nylon net filter with the *Vacuum Filter Apparatus* (see *Figure 9*). To ensure quantitative transfer of the *Sample solution* and any insoluble matter on the inner wall of the glass beaker, rinse the glass beaker twice with 200 mL of *Diluent* and pour the rinsing through the nylon net filter. Perform a final washing with a spray of water on the inner wall of the beaker and simultaneously pour this volume into the funnel. Wash the inner wall of the clamped funnel and the nylon net filter once with 200 mL of *Diluent* and twice with 250 mL water to ensure no insoluble matter remains on the inner wall of the funnel and no crystal residues of the tested amino acid are found on the nylon net

\(^4\) Millipore catalog number NY1H04700, or equivalent.
filter. Observe the residue on the nylon net filter with a magnifying glass (4×) with the aid of light to detect presence of any fibers. Measure the length of any fibers present, in mm. Carefully transfer the nylon net filter with any foreign matter that it may hold into a glass beaker that has been previously dried at 90° for 1 h and cooled in a desiccator for 1 h. Dry the beaker and nylon net filter at 90° for 1 h, and cool in a desiccator for 1 h. Weigh the nylon net filter with any foreign matter to the nearest 0.1 mg \( (X_2) \). Calculate the foreign matter weight:

\[
\text{Result} = X_2 - X_1
\]

\[
X_1 = \text{initial filter membrane weight (mg)}
\]

\[
X_2 = \text{final filter membrane weight (mg)}
\]

**Loss on Drying**

This procedure is used to determine the amount of volatile matter expelled under the conditions specified in the monograph. Because the volatile matter may include material other than adsorbed moisture, this test is designed for compounds in which the loss on drying may not definitely be attributable to water alone. For substances appearing to contain water as the only volatile constituent, the Direct (Karl Fischer) Titration Method, provided under Water, Appendix II B, is usually appropriate.

**PROCEDURE**

Unless otherwise directed in the monograph, conduct the determination on 1–2 g of the substance, previously mixed and accurately weighed. If the sample is in the form of large crystals, reduce the particle size to about 2 mm, quickly crushing the sample to avoid absorption or loss of moisture. Tare a glass-stoppered, shallow weighing bottle that has been dried for 30 min under the same conditions to be used in the determination. Transfer the sample to the bottle, replace the cover, and weigh the bottle and its contents. By gentle sideways shaking, distribute the sample as evenly as possible to a depth of about 5 mm for most substances and not over 10 mm in the case of bulky materials. Place the loaded bottle in the drying chamber, removing the stopper and leaving it also in the chamber, and dry at the temperature and for the length of time specified in the monograph. Upon opening the chamber, close the bottle promptly and allow it to come to room temperature, preferably in a desiccator, before weighing.

Where drying in vacuum is specified in the monograph, use a pressure as low as that obtainable by an aspirating water pump (NMT 20 mm Hg). If the test substance melts at a temperature lower than that specified for the determination, preheat the bottle and its contents for 1–2 h at a temperature 5°–10° below the melting range, then continue drying at the specified temperature for the determination. When drying the sample in a desiccator, ensure that the desiccant is kept fully effective by replacing it frequently.

**Nuclear Magnetic Resonance**

Nuclear magnetic resonance (NMR) spectroscopy is an analytical procedure based on the magnetic properties of certain atomic nuclei. It is similar to other types of spectroscopy in that absorption or emission of electromagnetic energy at characteristic frequencies provides analytical information. NMR differs in that the discrete energy levels between which the transitions take place are created artificially by placing the nuclei in a magnetic field.

Atomic nuclei are charged and behave as if they were spinning on the nuclear axis, thus creating a magnetic dipole of moment \( \mu \) along this axis. The angular momentum of the spinning nucleus is characterized by a spin quantum number \( I \). If the mass number is odd, \( I = \frac{1}{2} \) or an integer plus \( \frac{1}{2} \); otherwise, it has a value of 0 or a whole number.

Nuclei having a spin quantum number \( I \neq 0 \), when placed in an external uniform static magnetic field of strength, \( H_0 \), align with respect to the field in \( (2I + 1) \) possible orientations. Thus, for nuclei with \( I = \frac{1}{2} \), which include most isotopes of analytical significance, as shown in the table below, there are two possible orientations, corresponding to two different energy states. A nuclear resonance is the transition between these states, by absorption or emission of the corresponding amount of energy. In a static magnetic field the nuclear magnetic axis precesses (Larmor precession) about the external field axis. The precessional angular velocity, \( \omega_0 \), is related to the external magnetic field strength through the equation:

\[
\omega_0 = \gamma H_0
\]

in which \( \gamma \) is the magnetogyric ratio and is a constant for all nuclei of a given isotope. If energy from an oscillating radio-frequency field is introduced, the absorption of radiation takes place according to the relationship:

\[
\Delta E = h\nu = \mu H_0/I
\]

where \( h \) is Planck's constant, and

\[
\nu = \omega_0/2\pi = \gamma H_0/2\pi
\]

Thus, when the frequency \( (\nu_0) \) of the external energy field \( (E = h\nu) \) is the same as the precessional angular velocity, resonance is achieved.

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The energy difference between the two levels corresponds to electromagnetic radiation in the radio-frequency range. It is a function of \( \gamma \), which is a property of the nucleus, and \( H_0 \), the external field strength. As shown in the table below, the resonance frequency of a nucleus increases with the increase of the magnetic field strength.

NMR is a technique of high specificity but relatively low sensitivity. The basic reason for the low sensitivity is the comparatively small difference in energy between the excited and the ground states (0.02 calories at 15–20 kilogauss field strength), which results in a population difference between the two levels of only a few ppm. Another important aspect of the NMR phenomenon, with negative effects on the sensitivity, is the long lifetime of most nuclei in the excited state, which affects the design of the NMR analytical test, especially in pulsed repetitive experiments. Simultaneous acquisition of the entire spectrum instead of frequency-swept spectra can give sensitivity enhancement.

### Properties of Some Nuclei Amenable to NMR Study

<table>
<thead>
<tr>
<th>Nucleus</th>
<th>I</th>
<th>Natural Abundance, %</th>
<th>Sensitivity</th>
<th>1.4093 T*</th>
<th>2.3488 T</th>
<th>4.6975 T</th>
</tr>
</thead>
<tbody>
<tr>
<td>(^1\text{H})</td>
<td>(\frac{1}{2})</td>
<td>99.980</td>
<td>1.000</td>
<td>60.000</td>
<td>100.000</td>
<td>200.000</td>
</tr>
<tr>
<td>(^13\text{C})</td>
<td>(\frac{1}{2})</td>
<td>1.108</td>
<td>0.0159</td>
<td>15.087</td>
<td>25.144</td>
<td>50.288</td>
</tr>
<tr>
<td>(^19\text{F})</td>
<td>(\frac{1}{2})</td>
<td>100.000</td>
<td>0.830</td>
<td>56.446</td>
<td>94.077</td>
<td>188.154</td>
</tr>
<tr>
<td>(^31\text{P})</td>
<td>(\frac{1}{2})</td>
<td>100.000</td>
<td>0.0663</td>
<td>24.289</td>
<td>40.481</td>
<td>80.961</td>
</tr>
<tr>
<td>(^11\text{B}) ((\frac{1}{2}))</td>
<td>80.420</td>
<td>0.170</td>
<td>19.250</td>
<td>32.084</td>
<td>64.167</td>
<td></td>
</tr>
</tbody>
</table>

* T = tesla, 1 T = 10,000 Gauss.

### APPARATUS

The distinctive components of an NMR spectrometer are a magnet and a source of radio frequency. The instruments are described by the approximate resonance frequency of the analytical nucleus, e.g., \(^1\text{H}\) NMR. More recently, instruments are being referred to by their field strengths. Some spectrometers are dedicated to the analysis of one type of nucleus; others are designed to obtain spectra of different nuclei.

There are two types of commercial NMR spectrometers: the classical continuous wave (CW) instruments and the more modern pulse Fourier-transform (FT) instruments. The CW spectrometers use a technique similar to that of classical optical spectrometers: a slow scan of the radio frequency (at fixed magnetic field) or the magnetic field (at fixed radio frequency) over a domain corresponding to the resonance of the nuclei being studied. The signal generated by the absorption of energy is detected, amplified, and recorded.

Various instrument configurations are possible. The arrangement of a typical double-coil spectrometer, as one might see in the lower resolution 60-MHz and 100-MHz CW instruments, is illustrated in Figure 10.

![Figure 10. Block Diagram of a Typical NMR Spectrometer](image)

The limitations of the CW spectrometers are low sensitivity and long analysis time. In pulsed NMR spectrometers, a single pulse of radio frequency energy is used to simultaneously activate all nuclei. The excited nuclei returning to the lower energy level generate a free induction decay (FID) signal that contains in a time domain all the information obtained in a frequency domain with a CW spectrometer. The time domain and the frequency domain responses form a pair of FTs; the mathematical operation is performed by a computer after analog-to-digital conversion. After a delay allowing for relaxation of the excited nuclei, the pulse experiment (transient) may be repeated and the response coherently added in the computer memory, with random noise being averaged out. (A similar signal-to-noise increase can be obtained by combining CW spectrometers with computers that average transients.)

The block diagram of a typical high-resolution pulsed spectrometer is shown in Figure 11.
It is a typical configuration of the high-resolution spectrometer that uses a superconducting (cryogenic) solenoid as the source of the magnetic field. Introduction of the pulsed NMR spectrometer has made the acquisition of spectra of many nuclei, other than protons, routine. It has also allowed proton spectra to be obtained in much less time, and with smaller amounts of specimen, as compared to CW techniques.

NMR spectrometers have strict stability and homogeneity requirements. Stability is often achieved by a field-frequency locking system that “locks” the magnetic field to the resonance frequency of a reference signal. The lock signal can be homonuclear or heteronuclear. In the latter case, the reference resonance is usually a deuterium signal from a deuterated solvent. On older spectrometers, using deuterium as a locking nucleus permits noise decoupling of protons to be carried out while studying nuclei like $^{13}$C. While internal homonuclear locks are still used in CW proton spectrometers (where tetramethylsilane at about 0.5% provides a convenient lock), they are hardly ever used in pulsed FT spectrometers.

No type of magnet is capable of producing a homogeneous field over the space occupied by the specimen. Two techniques are usually employed to compensate for this lack of homogeneity: specimen spinning and the use of additional (shim) coils. Because of design, particularly probe design, the spinning in the case of the electromagnet or permanent magnet is perpendicular to the basic field. In the superconducting magnet, the axis of rotation can only be parallel to the basic magnetic field. The spin rate should be sufficient to produce averaging of the field, but not fast enough to produce an extended vortex in the specimen tube. A vortex extended near the region exposed to the radio-frequency coils decreases resolution. The shim coils are adjusted by the operator until instrumental contributions to the observed line width are minimized.

An electronic integrator is a feature of most NMR spectrometers. On a CW instrument ($^1$H and $^{19}$F) the integrator, connected to the spectrometer output stage, determines the relative areas of the resonance peaks and presents these areas as a series of stepped horizontal lines when a sweep is made in the integration mode. On FT-NMR spectrometers, an integration algorithm is included in the spectrometer software, and the resonance peak areas may be presented graphically as stepped lines or tabulated as numeric values. The use of computer-generated tabulated/numeric integration data should not be accepted without a specific demonstration of precision and accuracy on the spectrometer in question.

**THE SPECTRUM**

The signals (peaks) in an NMR spectrum are characterized by four attributes: resonance frequency, multiplicity, line width, and relative intensity. The analytical usefulness of the NMR technique resides in the fact that the same types of nuclei, when located in different molecular environments, exhibit different resonance frequencies. The reason for this difference is that the effective field experienced by a particular nucleus is a composite of the external field provided by the instrument and the field generated by the circulation of the surrounding electrons. (The latter is generally opposed to the external field and the phenomenon is called “shielding.”) In contrast with other spectroscopic methods, it is not possible to measure accurately the absolute values of transition frequencies. However, it is possible to measure accurately the difference in frequencies between two resonance signals. The position of a signal in an NMR spectrum is described by its separation from another resonance signal arbitrarily taken as standard. This separation is called chemical shift.

The chemical shift, being the difference between two resonance frequencies, is directly proportional to the magnetic field strength (or to the frequency of the oscillator). However, the ratio between the chemical shift, in frequency units, and the instrument frequency is constant. This allows definition of a dimensionless chemical shift parameter ($\delta$) that is independent of the instrument frequency:

$$\delta = (\nu_r - \nu_i)/\nu_i + \delta_i,$$

in which $\nu_r$ is the test substance line frequency, $\nu_i$ is the reference line frequency, $\nu_i$ is the instrument frequency, in mHz, and $\delta_i$ is the chemical shift of the reference.

By employing the above equation, it is possible to use (with appropriate caution) the chemical shift of any known species (such as the residual $^1$H-containing species in deuterated solvent) as a chemical shift reference. The above equation, now in common use, is applicable to nearly all methods except in the relatively rare cases where extremely precise chemical shift values must be determined, and is readily adaptable to nuclei where non-zero reference standards are the only practical method of chemical shift determinations.

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**Figure 11. Block Diagram of a Typical Pulsed FT-NMR Spectrometer**

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For CW instruments, tetramethylsilane (TMS) is the most widely used chemical shift reference for proton and carbon spectra. It is chemically inert, exhibits only one line, which is at a higher field than most signals, and is volatile, thus allowing for ready specimen recovery. Sodium 3-(trimethylsilyl)propionate (TSP) or sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) are used as NMR references for aqueous solutions. The resonance frequency of the TSP or DSS methyl groups closely approximate that of the TMS signal; however, DSS has the disadvantage of showing a number of methylene multiplets that may interfere with signals from the test substance. Where the use of an internal NMR reference material is not desirable, an external reference may be used.

Conventional NMR spectra are shown with the magnetic field strength increasing from left to right. Nuclei that resonate at high magnetic field strengths (to the right) are said to be more shielded (greater electron density) than those that resonate at lower magnetic field strengths: these are said to be de-shielded (lower electron density).

Figure 12 shows the proton NMR spectrum of 2,3-dimethyl-2-butenyl methyl ether. This compound contains protons in a methylene group (marked d in the graphic formula) and in four methyl groups (a, a, b, and c). Methyl groups b and c are situated in distinctly different molecular environments than the two a methyl groups. Three different methyl proton resonances are observed as spectral peaks in addition to the peak corresponding to methylene proton resonance. The two a methyl groups, being in very similar environments, have the same chemical shift. Interaction between magnetically active nuclei situated within a few bond lengths of each other leads to coupling, which results in a mutual splitting of the respective signals into sets of peaks or multiplets.

Figure 12. NMR Spectrum of 2,3-Dimethyl-2-butenyl methyl ether (15% in CCl₄) showing four nonequivalent, apparently uncoupled protons with a normal integral trace (peak area ratio from low H₀ to high H₀ of 2:3:3:6). (Tetramethylsilane, the NMR Reference, appears at 0 ppm.) The system of units represented by δ is defined under The Spectrum, in this section.

The coupling between two nuclei may be described in terms of the spin-spin coupling constant, J, which is the separation (in hertz) between the individual peaks of the multiplet. Where two nuclei interact and cause reciprocal splitting, the measured coupling constants in the two resulting mutiplets are equal. Furthermore, J is independent of magnetic field strength.

In a first-order, comparatively noncomplex spin system, the number of individual peaks that are expected to be present in a multiplet and the relative peak intensities are predictable. The number of peaks is determined by 2 n₁ + 1, where n is the number of nuclei on adjacent groups that are active in splitting. For protons this becomes (n + 1) peaks. In general, the relative intensity of each peak in the multiplet follows the coefficient of the binomial expansion \((a + b)^n\). These coefficients may conveniently be found by use of Pascal’s triangle, which produces the following relative areas for the specified multiplets: doublet, 1:1; triplet, 1:2:1; quartet, 1:3:3:1; quintet, 1:4:6:4:1; sextet, 1:5:10:10:5:1; and septet, 1:6:15:20:15:6:1. This orderly arrangement, generally referred to as first-order behavior, may be expected when the ratio of \(D_v\) to \(J\) is greater than about 10; \(D_v\) is the chemical shift difference between two nuclei or two groups of equivalent nuclei. Two examples of idealized spectra arising from first-order coupling are shown in Figure 13.
Figure 13. Diagrammatic Representation of Simple First-Order Coupling of Adjacent Protons

Figure 14 shows a spectrum displaying triplet signals resulting from the mutual splitting of two adjacent methylene groups.

Figure 14. NMR Spectrum of 3-Keto-tetrahydrofuran (10% in CCl₄) showing three nonequivalent protons, with a normal integral trace (peak area ratio from low Hₐ to high Hₐ of 1:1:1). Note two sets of methylene groups coupled to each other at 4.2 and 2.4 ppm. (Tetramethylsilane, the NMR Reference, appears at 0 ppm.)

Coupling may occur between ¹H and other nuclei, such as ¹⁹F, ¹³C, and ³¹P. In some cases, e.g., in the CW mode, the coupling constants may be large enough so that part of the multiplet is off scale at either the upfield or downfield end. This type of coupling may occur over the normal “three-bond distance,” as for ¹H-¹H coupling.

Magnetically active nuclei with I ≥ 1, such as ¹⁴N, possess an electrical quadrupole moment, which produces line-broadening of the signal due to neighboring nuclei.

Another characteristic of the signal, its relative intensity, has wide analytical applications. In carefully designed experiments (see General Method, below), the area or intensity of a signal is directly proportional to the number of protons giving rise to the signal. As a result, it is possible to determine the relative ratio of the different kinds of protons or other nuclei in a specimen or to perform NMR assays with the aid of an internal standard.
The NMR spectra may contain extraneous signals due to the inhomogeneity of the magnetic field throughout the specimen. These artifacts, called spinning side bands, appear as minor lines symmetrically located around each signal. The presence of large spinning side bands indicates that the non-spinning shims require adjustment. The separation is equal to the frequency of the specimen tube spin rate or some integral multiple of that frequency. Thus, spinning side bands are readily identifiable.

**GENERAL METHOD**

Inadequate specimen preparation or incorrect instrumental adjustments and parameters may lead to poor resolution, decreased sensitivity, spectral artifacts, and erroneous data. It is preferable that the operator be familiar with the basic theory of NMR, the properties of the specimen, and the operating principles of the instruments. Strict adherence to the instruction manuals provided by the manufacturer and frequent checks of the performance of the instrument are essential.

The method and procedures discussed here refer specifically to $^1$H (proton) and $^{19}$F NMR. They are applicable, with modification, to other nuclei. The discussion presumes that the NMR spectra are obtained from liquid test substances or solutions in suitable solvents.

**SELECTION OF SOLVENT**

In addition to having good solubility properties, suitable solvents do not exhibit resonance peaks that obscure resonance peaks of the specimen being analyzed. The most commonly used solvents for proton and carbon NMR are listed in the table below. Deuterated solvents also provide the signal for the heteronuclear system lock. If solvent peaks might interfere with any signals from the specimen, then the isotopic purity of the solvent should be as high as possible. Deuterium (I = 1) does not exhibit resonance under $^1$H conditions but may cause J-coupling to be observed. The residual protons generate solvent peaks whose chemical shifts are shown in the table below.

<table>
<thead>
<tr>
<th>Solvents Commonly Used for Proton NMR</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Solvent</strong></td>
</tr>
<tr>
<td>CCl$_4$</td>
</tr>
<tr>
<td>CS$_2$</td>
</tr>
<tr>
<td>SO$_2$ (liquid)</td>
</tr>
<tr>
<td>(CF$_3$)$_2$CO</td>
</tr>
<tr>
<td>CDCl$_3$</td>
</tr>
<tr>
<td>CD$_3$OD</td>
</tr>
<tr>
<td>(CD$_3$)$_2$CO</td>
</tr>
<tr>
<td>D$_2$O</td>
</tr>
<tr>
<td>DMSO-d$_6$</td>
</tr>
<tr>
<td>C$_6$D$_6$</td>
</tr>
<tr>
<td>p-Dioxane-d$_4$</td>
</tr>
<tr>
<td>CD$_3$COOD</td>
</tr>
<tr>
<td>DMF-d$_7$</td>
</tr>
</tbody>
</table>

$^a$ $\delta$ in ppm relative to tetramethylsilane arbitrarily taken as 0 or 0 ppm.

$^b$ Spectrophotometric grade.

$^c$ Highly variable; depends on solute and temperature.

$^d$ Dimethyl sulfoxide-d$_6$.

$^e$ N,N-Dimethylformamide-d$_6$ per Aldrich, Alfa, Fluka, and Sigma catalogs.

Some solvents (e.g., D$_2$O or CD$_3$OD) enter into fast exchange reactions with protons and may eliminate resonance signals from –COOH, –OH, and –NH$_2$ structural groups. The protons in alcohols and amines do not take part in rapid exchange unless catalyzed by small concentrations of acid or base, except in the presence of D$_2$O and some other solvents (e.g., CD$_3$OD).

For $^{19}$F NMR, most solvents used in proton NMR may be employed, the most common ones being CHCl$_3$, CCl$_4$, H$_2$O, CS$_2$, aqueous acids and bases, and dimethylacetamide. In general, any nonfluorinated solvent may be used, provided that it is of spectral quality. Obviously, there is no interference from the protonated functional groups of the solvent. However, unless they are decoupled, protonated functional groups on the $^{19}$F-containing specimen will provide J-coupling.
SPECIMEN PREPARATION

Directions are usually given in individual monographs. The solute concentration depends on the objective of the experiment and on the type of instrument. Detection of minor contaminants may require higher concentrations. The solutions are prepared in separate vials and transferred to the NMR specimen tube. The volume required depends on the size of the specimen tube and on the geometry of the instrument. The level of the solution in the tube must be high enough to extend beyond the coils when the tube is inserted in the instrument probe and spun.

The NMR specimen tubes must meet narrow tolerance specifications in diameter, wall thickness, concentricity, and camber. The most widely used tubes have a 5-mm or 10-mm outside diameter and a length of 15–20 cm. Microtubes are available for the analysis of small amounts of specimen.

PROCEDURE

The specimen tube is placed in a probe located in the magnetic field. The probe contains electronic circuitry including the radio-frequency coil(s), and is provided with attachments for the air supply that spins the specimen tubes.

Instrument adjustments are made before each experiment. The spinning rate of the specimen tube is adjusted so that spinning side bands do not interfere with the peaks of interest and the vortex does not extend beyond the coils in the probe.

To optimize the instrument performance, the magnetic shim gradients on FT-NMR spectrometers are adjusted. In adjusting resolution on CW spectrometers, a good indicator is the definite "ringing" of the TMS peak. The phenomenon of ringing is the oscillation of the recorder trace after the magnetic field has passed through a resonance frequency. Ringing, evident on a number of the peaks in Figures 14 and 15, arises during rapid scans and decays exponentially to the baseline value.

![Figure 15. Continuous Wave Proton Spectrum of Ethyl Ether](image)

*Figure 15. Continuous Wave Proton Spectrum of Ethyl Ether*

*Figure 16 clearly indicates the absence, in an FT experiment, of the ringing phenomenon. Ringing will not appear because the spectrum obtained is the result of analysis of the FID by Fourier transformation and not a magnetic field or frequency sweep through the individual resonance positions.*

![Figure 16. Proton NMR Spectrum of Ethyl Ether in Deuterated Chloroform](image)

*Figure 16. Proton NMR Spectrum of Ethyl Ether in Deuterated Chloroform*
With proton CW instruments the spectrum is scanned from 0 ppm to about 10 ppm with a scan time of about 1–5 min. The amplification is adjusted so that all peaks remain on scale. If the response is low at reasonable amplitude, the radio-frequency power is increased to obtain the highest possible peak response without peak broadening. After the initial scan, the presence of peaks downfield of 10 ppm is quickly checked by offsetting the instrument response by about 5 ppm. With CW instrumentation, it is common for the TMS peak to shift slightly during an extended scan. The extent of the shift is usually obtained by comparing the relative positions of another peak in the initial scan with the same peak in the offset scan.

The operation of an FT-NMR spectrometer is a much more elaborate experiment. The computer serves to control the spectrometer, to program the experiment, and to store and process the data. Programming the experiment involves setting values for a large number of variables including the spectral width to be examined, the duration (“width”) of the excitation pulse, the time interval over which data will be acquired, the number of transients to be accumulated, and the delay between one acquisition and the next. The analysis time for one transient is in the order of seconds. The number of transients is a function of the specimen concentration, the type of nucleus, and the objective of the experiment. At the end of the experiment, the FID signal is stored in digitized form in the computer memory and is displayed on the video screen. The signal can be processed mathematically to enhance either the resolution or the sensitivity, and it can be Fourier-transformed into a frequency-domain spectrum. The instrument provides a plot of the spectrum. The integration routine, accessed through keyboard commands, results in a stepped-line plot. Considerably more accurate integrals are obtained if the signals or regions of interest are separately integrated.

FT-NMR spectrometers may yield qualitative and quantitative data from the same experiment, but this is seldom done in practice. In quantitative FT experiments, special precautions must be taken for the signal areas to be proportional to the number of protons. The delays between pulses must be long enough to allow complete relaxation of all excited nuclei. This results in a considerable increase in analysis time and in some loss of resolution. Qualitative analysis is usually performed in nonquantitative conditions, with the design of the experiment directed to fast analysis with maximum resolution or sensitivity.

QUALITATIVE AND QUANTITATIVE ANALYSIS

NMR spectroscopy has been used for a wide range of applications such as structure elucidation; thermodynamic, kinetic, and mechanistic studies; and quantitative analysis. Some of these applications are beyond the scope of compendial methods.

All five characteristics of the signal (chemical shift, multiplicity, line width, coupling constants, and relative intensity) contribute analytical information.

QUALITATIVE APPLICATIONS

Comparison of a spectrum from the literature or from an authentic specimen with that of a test specimen may be used to confirm the identity of a compound and to detect the presence of impurities that generate extraneous signals. The NMR spectra of simple structures can be adequately described by the numeric value of the chemical shifts and coupling constants, and by the number of protons under each signal. (The software of modern instruments includes programs that generate simulated spectra using these data.) Experimental details, such as the solvent used, the specimen concentration, and the chemical shift reference, must also be provided.

For unknown specimens, NMR analysis, usually coupled with other analytical techniques, is a powerful tool for structure elucidation. Chemical shifts provide information on the chemical environment of the nuclei. Extensive literature is available with correlation charts and rules for predicting chemical shifts. The multiplicity of the signals provides important stereochemical information. Mutual signal splitting of functional groups indicates close proximity. The magnitude of the coupling constant, J, between residual protons on substituted aromatic, olefinic, or cycloalkyl structures is used to identify stereochemical information. The presence of peaks downfield of 10 ppm is quickly checked by offsetting the instrument response by about 5 ppm. With CW instrumentation, it is common for the TMS peak to shift slightly during an extended scan. The extent of the shift is usually obtained by comparing the relative positions of another peak in the initial scan with the same peak in the offset scan.

The operation of an FT-NMR spectrometer is a much more elaborate experiment. The computer serves to control the spectrometer, to program the experiment, and to store and process the data. Programming the experiment involves setting values for a large number of variables including the spectral width to be examined, the duration (“width”) of the excitation pulse, the time interval over which data will be acquired, the number of transients to be accumulated, and the delay between one acquisition and the next. The analysis time for one transient is in the order of seconds. The number of transients is a function of the specimen concentration, the type of nucleus, and the objective of the experiment. At the end of the experiment, the FID signal is stored in digitized form in the computer memory and is displayed on the video screen. The signal can be processed mathematically to enhance either the resolution or the sensitivity, and it can be Fourier-transformed into a frequency-domain spectrum. The instrument provides a plot of the spectrum. The integration routine, accessed through keyboard commands, results in a stepped-line plot. Considerably more accurate integrals are obtained if the signals or regions of interest are separately integrated.

FT-NMR spectrometers may yield qualitative and quantitative data from the same experiment, but this is seldom done in practice. In quantitative FT experiments, special precautions must be taken for the signal areas to be proportional to the number of protons. The delays between pulses must be long enough to allow complete relaxation of all excited nuclei. This results in a considerable increase in analysis time and in some loss of resolution. Qualitative analysis is usually performed in nonquantitative conditions, with the design of the experiment directed to fast analysis with maximum resolution or sensitivity.

QUALITATIVE AND QUANTITATIVE ANALYSIS

NMR spectroscopy has been used for a wide range of applications such as structure elucidation; thermodynamic, kinetic, and mechanistic studies; and quantitative analysis. Some of these applications are beyond the scope of compendial methods.

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The software of modern FT-NMR spectrometers allows for sequences of pulses much more complex than the repetitive accumulation of transients described above. Such experiments include homonuclear or heteronuclear two-dimensional analysis, which determines the correlation of couplings and may simplify the interpretation of otherwise complex spectra.

QUANTITATIVE APPLICATIONS

If appropriate instrument settings for quantitative analysis have been made, the areas (or intensities) of two signals are proportional to the total number of protons generating the signals.

\[ \frac{A_1}{A_2} = \frac{N_1}{N_2} \]  

(1)

If the two signals originate from two functional groups of the same molecule, the equation can be simplified to:

\[ \frac{A_1}{A_2} = \frac{n_1}{n_2} \]  

(2)

in which \( n_1 \) and \( n_2 \) are the number of protons in the respective functional groups.

If the two signals originate from different molecular species,

\[ \frac{A_1}{A_2} = \frac{n_1 m_1}{n_2 m_2} = \frac{(n_1 W_1 / M_1)}{(n_2 W_2 / M_2)} \]  

(3)

in which \( m_1 \) and \( m_2 \) are the numbers of moles; \( W_1 \) and \( W_2 \) are the masses; and \( M_1 \) and \( M_2 \) are the molecular weights of compounds 1 and 2, respectively.

Examination of Equations 2 and 3 shows that NMR quantitative analysis can be performed in an absolute or relative manner. In the absolute method, an internal standard is added to the specimen and a resonance peak area arising from the test substance is compared with a resonance peak area from the internal standard. If both test substance and internal standard are accurately weighed, the absolute purity of the substance may be calculated. A good internal standard has the following properties: it presents a reference resonance peak, preferably a singlet, at a field position removed from all specimen peaks; it is soluble in the analytical solvent; its proton equivalent weight, i.e., the molecular weight divided by the number of protons giving rise to the reference peak, is low; and it does not interact with the compound being tested. Typical examples of useful standards are 1,2,4,5-tetrachlorobenzene, 1,4-dinitrobenzene, benzyl benzoate, and maleic acid. The choice of a standard will be dictated by the spectrum of the specimen.

The relative method may be used to determine the molar fraction of an impurity in a test substance (or of the components in a mixture) as calculated by Equation 3.

Quantitative analysis, as well as detection of trace impurities, is markedly improved with modern instrumentation. Stronger magnetic fields and the ability to accumulate and/or average signals over long periods of time greatly enhance the sensitivity of the method.

ABSOLUTE METHOD OF QUANTITATION

Where the individual monograph directs that the Absolute Method of Quantitation be employed, proceed as follows.

**Solvent, Internal standard, and NMR reference:** Use as directed in the individual monograph.

**Test preparation:** Transfer an accurately weighed quantity of the test substance, containing about 4.5 proton mEq, to a glass-stoppered, graduated centrifuge tube. Add about 4.5 proton mEq of Internal standard, accurately weighed, and 3.0 mL of Solvent, insert the stopper, and shake. When dissolution is complete, add about 30 µL (30 mg if a solid) of NMR Reference, provided that it does not interfere with subsequent measurements, and shake.

**Procedure:** Transfer an appropriate amount (0.4–0.8 mL) of Test preparation to a standard 5-mm NMR spinning tube, and record the spectrum, adjusting the spin rate so that no spinning side bands interfere with the peaks of interest. Measure the area under each of the peaks specified in the individual monograph by integrating not fewer than five times. Record the average area or intensity resulting from the resonances of the groups designated in the individual monograph as \( A_1 \) and \( A_2 \).

Calculate the quantity, in mg, of the analyte in the Test preparation:

\[ W_1 (A_1 / A_2) (E_2 / E_1) \]

in which \( W_1 \) is the weight, in mg, of Internal standard taken; and \( E_2 \) and \( E_1 \) are the proton equivalent weights (i.e., the molecular weights divided by the number of protons giving rise to the reference peak) of the analyte and the Internal standard, respectively.

RELATIVE METHOD OF QUANTITATION

Where the individual monograph directs that the Relative Method of Quantitation be employed, proceed as follows.

**Solvent, NMR reference, and Test preparation:** Use as directed under Absolute Method of Quantitation.

**Procedure:** Transfer an appropriate amount (0.4–0.8 mL) of Test preparation to a standard 5-mm NMR spinning tube, and record the spectrum, adjusting the spin rate so that no spinning side bands interfere with the peaks of interest. Measure the area or intensity under each of the peaks specified in the individual monograph by integrating not fewer than five times. Record the average area or intensity resulting from the resonances of the groups designated in the individual monograph as \( A_1 \) and \( A_2 \).

Calculate the quantity, in mole percent, of the analyte in the Test preparation:
\[
(100 \times (A_1/n_1)/[(A_1/n_1) + (A_2/n_2)]
\]

in which \(n_1\) and \(n_2\) are, respectively, the numbers of protons in the designated groups.

**Oil Content of Synthetic Paraffin**

**APPARATUS**

**Filter stick:** Use either a 10-mm diameter sintered-glass filter stick of 10–15-µm maximum pore diameter, or a filter stick made of stainless steel and having a 0.5-in. disk of 10–15-µm maximum pore diameter. Determine conformance with the pore diameter specified as follows: Clean sintered-glass filter sticks by soaking in hydrochloric acid, or stainless steel sticks by soaking in nitric acid, wash with water, rinse with acetone, and dry in air followed by drying in an oven at 105° for 30 min.

Thoroughly wet the clean filter stick by soaking in water, and then connect it with an apparatus (see Figure 17) consisting of a mercury-filled manometer, readable to 0.5 mm; a clean and filtered air supply; a drying bulb filled with silica gel; and a needle-valve type air pressure regulator. Apply pressure slowly from the air source, and immerse the filter just below the surface of water contained in a beaker.

[Figure 17. Assembly for Checking Pore Diameter of Filter Sticks]

[NOTE—If a head of liquid is noted above the surface of the filter after it is inserted into the water, the back pressure thus produced should be subtracted from the observed pressure when the pore diameter is calculated as directed below.]

Increase the air pressure to 10 mm below the acceptable pressure limit, and then increase the pressure at a slow, uniform rate of about 3 mm Hg per minute until the first bubble passes through the filter. This can be conveniently observed by placing the beaker over a mirror. Read the manometer when the first bubble passes off the underside of the filter. Calculate the pore diameter, in µm:

\[
\text{Result} = 2180/p
\]

in which \(p\) is the observed pressure, in mm, corrected for any back pressure as mentioned above.

**Filtration assembly:** Connect the Filter Stick with an air pressure inlet tube and delivery nozzle and ground-glass joint to fit a 25-mm × 170-mm test tube as shown in Figure 18. If a stainless steel Filter Stick is used, make the connection to the test tube by means of a cork.
Cooling bath: Use a suitable insulated box having 1-in. holes in the center to accommodate any desired number of test tubes. The bath may be filled with a suitable medium such as kerosene and may be cooled by circulating a refrigerant through coils, or by using solid carbon dioxide, to produce a temperature of 30 ± 2°F.

Air pressure regulator: Use a suitable pressure-reduction valve, or other suitable regulator, that will supply air to the Filtration Assembly at the volume and pressure required to give an even flow of filtrate (see Procedure). Connect the regulator with rubber tubing to the end of the Filter Stick in the Filtration Assembly.

Thermometer: Use an ASTM Oil in Wax Thermometer having the range of −35° to +70°F and conforming to the requirements for an ASTM 71F thermometer (see Thermometers, Appendix I).

Weighing bottles: Use glass-stoppered conical bottles having a capacity of 15 mL. The bottles are used as evaporating flasks in the Procedure.

Evaporation assembly: The assembly consists of an evaporating cabinet capable of maintaining a temperature of 95 ± 2°F around the evaporation flasks, and air jets (4 ± 0.2 mm id) for delivering a stream of clean, dry air vertically downward into the flasks. In the Procedure below, support each jet so that the tip is 15 ± 5 mm above the surface of the liquid at the start of the evaporation. Supply the air (purified by passage through a tube of 1-cm bore packed loosely to a height of 20 cm with absorbent cotton) at the rate of 2 to 3 L/min per jet. The cleanliness of the air should be checked periodically to ensure that NMT 0.1 mg of residue is obtained when 4 mL of methyl ethyl ketone is evaporated as directed in the Procedure.

Wire stirrer: Use a 250-mm length of stiff iron or nichrome wire of about No. 20 B & S gauge. Form a 10-mm diameter loop at each end, and bend the loop at the bottom end so that the plane of the loop is perpendicular to the length of the wire.

SAMPLE SELECTION

If the sample weighs about 1 kg or less, obtain a representative portion by melting the entire sample and stirring thoroughly. For samples heavier than about 1 kg, exercise special care to ensure that a truly representative portion is obtained, noting that the oil may not be distributed uniformly throughout the sample and that mechanical operations may have expressed some of the oil.

PROCEDURE

Melt a representative portion of the sample in a beaker, using a water bath or oven maintained at 160°–210°F. As soon as the sample is completely melted, thoroughly mix it by stirring. Preheat a dropper pipet, provided with a rubber bulb and calibrated to deliver 1 ± 0.05 g of molten sample, and withdraw a 1-g portion of the sample as soon as possible after it has melted. Hold the pipet in a vertical position, and carefully transfer its contents into a clean, dry test tube previously weighed to the nearest milligram. Evenly coat the bottom of the tube by swirling, allow the tube to cool, and weigh to the nearest milligram. Calculate the sample weight, in grams, and record it as B (see Calculation). Pipet 15 mL of methyl ethyl ketone (ASTM Specification D 740, or equivalent) into the tube, and immerse the tube up to the top of the liquid in a hot water or steam bath. Stir with an up-and-down motion with the wire stirrer, and continue heating and stirring until a homogeneous solution is obtained, exercising care to avoid loss of solvent by prolonged boiling.

[Note—If it appears that a clear solution will not be obtained, stir until any undissolved material is well dispersed so as to produce a slightly cloudy solution.]

Figure 18. Filtration Assembly for Determination of Oil Content
After the sample solution is prepared, plunge the test tube into an 800-mL beaker of ice water, and continue to stir until the contents are cold. Remove the stirrer, then remove the test tube from the bath, dry the outside of the tube with a cloth, and weigh to the nearest 10 mg. Calculate the weight, in grams, of solvent in the test tube, and record it as C (see Calculation). Place the tube in the cooling bath, maintained at −30 ± 2°F, and stir continuously with the thermometer until the temperature reaches −25 ± 0.5°F, maintaining the slurry at a uniform consistency and taking precautions to prevent the sample from setting up on the walls of the tube or forming crystals. Place the filter stick in a test tube and cool at −30 ± 2°F in the cooling bath for a minimum of 10 min. Immerse the cooled filter stick in the sample, then connect the filtration assembly, seating the ground-glass joint of the filter so as to make an airtight seal. Place an unstoppered weighing bottle, previously weighed together with the glass stopper to the nearest 0.1 mg, under the delivery nozzle of the filtration assembly.

[Note—Suitable precautions and proper analytical technique should be applied to ensure the accuracy of the weight of the bottle. Before determining its weight, the bottle and its stopper should have been cleaned and dried, then rinsed with methyl ethyl ketone, wiped dry on the outside, dried in the evaporation assembly for about 5 min, and cooled. Then allow it to stand for about 10 min near the balance before weighing.]

Apply air pressure to the filtration assembly, immediately collect about 4 mL of filtrate in the weighing bottle, and release the air pressure to permit the liquid to drain back slowly from the delivery nozzle. Stopper the bottle, and weigh it to the nearest 10 mg without waiting for it to come to room temperature. Remove the stopper, transfer the bottle to the evaporation assembly maintained at 95 ± 2°F, and place it under an air jet centered inside the neck, with the tip 15 ± 5 mm above the surface of the liquid. After the solvent has evaporated (usually less than 30 min), stopper the bottle, and allow it to stand near the balance for about 10 min before it is weighed to the nearest 0.1 mg. Repeat the evaporation procedure for 5-min periods until the loss between successive weighings is NMT 0.2 mg. Determine the weight of the oil residue, in grams, by subtracting the weight of the empty stoppered bottle from the weight of the stoppered bottle plus the oil residue after the evaporation procedure, and record the results as A (see Calculation). Determine the weight of solvent evaporated, in grams, by subtracting the weight of the bottle plus oil residue from the weight of the bottle plus filtrate, and record the result as D (see Calculation).

**CALCULATION**

Calculate the percent, by weight, of oil in the sample:

\[
\text{Result} = \frac{100 \times AC}{BD} - 0.15
\]

in which 0.15 is a factor to correct for solubility of the sample in the solvent at −25°F.

**Plasma Spectrochemistry**

Plasma-based instrumental techniques that are useful for food ingredient analyses fall into two major categories: those based on the inductively coupled plasma, and those where a plasma is generated at or near the surface of the sample. An inductively coupled plasma (ICP) is a high-temperature excitation source that desolvates, vaporizes, and atomizes aerosol samples and ionizes the resulting atoms. The excited analyte ions and atoms can then subsequently be detected by observing their emission lines, a method termed inductively coupled plasma–atomic emission spectroscopy (ICP–AES), also known as inductively coupled plasma–optical emission spectroscopy (ICP–OES); or the excited or ground state ions can be determined by a technique known as inductively coupled plasma–mass spectrometry (ICP–MS). ICP–AES and ICP–MS may be used for either single- or multi-element analysis, and they provide good general-purpose procedures for either sequential or simultaneous analyses over an extended linear range with good sensitivity.

An emerging technique in plasma spectrochemistry is laser-induced breakdown spectroscopy (LIBS). In LIBS, a solid, liquid, or gaseous sample is heated directly by a pulsed laser, or indirectly by a plasma generated by the laser. As a result, the sample is volatilized at the laser beam contact point, and the volatilized constituents are reduced to atoms, molecular fragments, and larger clusters in the plasma that forms at or just above the surface of the sample. Emission from the atoms and ions in the sample is collected, typically using fiber optics or a remote viewing system, and is measured using an array detector such as a charge-coupled device (CCD). LIBS can be used for qualitative analysis or against a working standard curve for quantitative analysis. Although LIBS is not currently in wide use, it might be suited for at-line or on-line measurements in a production setting, as well as in the laboratory. Because of its potential, it should be considered a viable technique for plasma spectrochemistry in the laboratory. However, because LIBS is still an emerging technique, details will not be further discussed here.\(^5\)

**SAMPLE PREPARATION**

Sample preparation is critical to the success of plasma-based analysis and is the first step in performing any analysis via ICP–AES or ICP–MS. Plasma-based techniques are heavily dependent on sample transport into the plasma, and because ICP–AES and ICP–MS share the same sample introduction system, the means by which samples are prepared may be applicable to either technique. The most conventional means by which samples are introduced into the plasma is via solution nebulization. If solution nebulization is employed, solid samples must be dissolved in order to be presented into the plasma for analysis. Samples may be dissolved in any appropriate solvent. There is a strong preference for the use of aqueous or

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dilute nitric acid solutions, because there are minimal interferences with these solvents compared to other solvent choices. Hydrogen peroxide, hydrochloric acid, sulfuric acid, perchloric acid, combinations of acids, or various concentrations of acids can all be used to dissolve the sample for analysis. Dilute hydrofluoric acid may also be used, but great care must be taken to ensure the safety of the analyst, as well as to protect the quartz sample introduction equipment when using this acid; specifically, the nebulizer, spray chamber, and inner torch tube should be manufactured from hydrofluoric-acid-tolerant materials. Additionally, alternative means of dissolving the sample can be employed. These include, but are not limited to, the use of dilute bases, straight or diluted organic solvents, combinations of acids or bases, and combinations of organic solvents.

When samples are introduced into the plasma via solution nebulization, it is important to consider the potential matrix effects and interferences that might arise from the solvent. The use of an appropriate internal standard and/or matching the standard matrix with samples should be applied for ICP–AES and ICP–MS analyses in cases where accuracy and precision are not adequate. In either event, the selection of an appropriate internal standard should consider the analyte in question, ionization energy, wavelengths or masses, and the nature of the sample matrix.

Where a sample is found not to be soluble in any acceptable solvent, a variety of digestion techniques can be employed. These include hot-plate digestion and microwave-assisted digestions, including open- and closed-vessel approaches. The decision regarding the type of digestion technique to use depends on the nature of the sample being digested, as well as on the analytes of interest.

Open-vessel digestion is generally not recommended for the analysis of volatile metals, e.g., selenium and mercury. The suitability of a digestion technique, whether open- or closed-vessel, should be supported by spike recovery experiments in order to verify that, within an acceptable tolerance, volatile metals have not been lost during sample preparation. Use acids, bases, and hydrogen peroxide of ultra-high purity, especially when ICP–MS is employed. Deionized water must be at least 18 megaohm. Check diluents for interferences before they are used in an analysis. Because it is not always possible to obtain organic solvents that are free of metals, use organic solvents of the highest quality possible with regard to metal contaminants.

It is important to consider the selection of the type, material of construction, pretreatment, and cleaning of analytical labware used in ICP–AES and ICP–MS analyses. The material must be inert and, depending on the specific application, resistant to caustics, acids, and/or organic solvents. For some analyses, diligence must be exercised to prevent the adsorption of analytes onto the surface of a vessel, particularly in ultra-trace analyses. Contamination of the sample solutions from metal and ions present in the container can also lead to inaccurate results.

The use of labware that is not certified to meet Class A tolerances for volumetric flasks is acceptable if the linearity, accuracy, and precision of the method have been experimentally demonstrated to be suitable for the purpose at hand.

### SAMPLE INTRODUCTION

There are two ways to introduce the sample into the nebulizer: by a peristaltic pump and by self-aspiration. The peristaltic pump is preferred, and serves to ensure that the flow rate of sample and standard solution to the nebulizer is the same, irrespective of sample viscosity. In some cases, where a peristaltic pump is not required, self-aspiration can be used.

A wide variety of nebulizer types is available, including pneumatic (concentric and cross-flow), grid, and ultrasonic nebulizers. Micronebulizers, high-efficiency nebulizers, direct-injection high-efficiency nebulizers, and flow-injection nebulizers are also available. The selection of the nebulizer for a given analysis should consider the sample matrix, analyte, and desired sensitivity. Some nebulizers are better suited for use with viscous solutions or those containing a high concentration of dissolved solids, whereas others are better suited for use with organic solutions.

Note that the self-aspiration of a fluid is due to the Bernoulli or Venturi effect. Not all types of nebulizers will support self-aspiration. The use of a concentric nebulizer, for example, is required for self-aspiration of a solution.

Once a sample leaves the nebulizer as an aerosol, it enters the spray chamber, which is designed to permit only the smallest droplets of sample solution into the plasma; as a result, typically only 1%–2% of the sample aerosol reaches the ICP, although some special-purpose nebulizers have been designed that permit virtually all of the sample aerosol to enter the ICP. As with nebulizers, there is more than one type of spray chamber available for use with ICP–AES or ICP–MS. Examples include the Scott double-pass spray chamber, as well as cyclonic spray chambers of various configurations. The spray chamber must be compatible with the sample and solvent, and must equilibrate and wash out in as short a time as possible. When a spray chamber is selected, the nature of the sample matrix, the nebulizer, the desired sensitivity, and the analyte should all be considered.

Gas and liquid chromatography systems can be interfaced with ICP–AES and ICP–MS for molecular speciation, ionic speciation, or other modes of separation chemistry, based on elemental emission or mass spectrometry.

Ultimately, the selection of sample introduction hardware should be demonstrated experimentally to provide sufficient specificity, sensitivity, linearity, accuracy, and precision of the analysis at hand.

In addition to solution nebulization, it is possible to analyze solid samples directly via laser ablation (LA). In such instances, the sample enters the torch as a solid aerosol. LA–ICP–AES and LA–ICP–MS are better suited for qualitative analyses of compounds, because of the difficulty in obtaining appropriate standards. Nonetheless, quantitative analyses can be performed if it can be demonstrated through appropriate method validation that the available standards are adequate.6

### STANDARD PREPARATION

Single- or multi-element standard solutions, which have concentrations traceable to primary reference standards, such as those of the National Institute of Standards and Technology (NIST), can be purchased for use in the preparation of working standard solutions. Alternatively, standard solutions of elements can be accurately prepared from standard materials and

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their concentrations, determined independently, as appropriate. Working standard solutions, especially those used for ultra-
trace analyses, may have limited shelf life. As a general rule, working standard solutions should be retained for no more than
24 h, unless stability is demonstrated experimentally. The selection of the standard matrix is of fundamental importance in
the preparation of element standard solutions. Spike recovery experiments should be conducted with specific sample
matrices in order to determine the accuracy of the method. If sample matrix effects cause excessive inaccuracies, standards,
blanks, and sample solutions should be matrix matched, if possible, in order to minimize matrix interferences.

In cases where matrix matching is not possible, an appropriate internal standard or the method of standard additions
should be used for ICP–AES or ICP–MS. Internal standards can also be introduced through a T connector into the sample
takeup tubing. In any event, the selection of an appropriate internal standard should consider the analytes in question, their
ionization and excitation energies, their chemical behavior, their wavelengths or masses, and the nature of the sample
matrix. Ultimately, the selection of an internal standard should be demonstrated experimentally to provide sufficient
spatial isotope, sensitivity, linearity, accuracy, and precision at the analyte concentration.

The method of standard additions involves adding a known concentration of the analyte element to the sample at no fewer than two concentration levels plus an unspiked sample preparation. The instrument response is plotted against the
concentration of the added analyte element, and a linear regression line is drawn through the data points. The absolute
value of the x-intercept multiplied by any dilution factor is the concentration of the analyte in the sample.

The presence of dissolved carbon at concentrations of a small percentage in aqueous solutions enhances ionization of
selenium and arsenic in an inductively coupled argon plasma. This phenomenon frequently results in a positive bias for ICP–
AES and ICP–MS selenium and arsenic quantification measurements, which can be remedied by using the method of
standard additions or by adding a small percentage of carbon, such as analytically pure glacial acetic acid, to the linearity
standards.

**ICP**

The components that make up the ICP excitation source include the argon gas supply, torch, radio frequency (RF)
induction coil, impedance-matching unit, and RF generator. Argon gas is almost universally used in an ICP. The plasma torch
consists of three concentric tubes designated as the inner, the intermediate, and the outer tube. The intermediate and outer
tubes are almost universally made of quartz. The inner tube can be made of quartz or alumina if the analysis is conducted
with solutions containing hydrofluoric acid. The nebulizer gas flow carries the aerosol of the sample solution into and
through the inner tube of the torch and into the plasma. The intermediate tube carries the intermediate (sometimes referred
to as the auxiliary) gas. The intermediate gas flow helps to lift the plasma off the inner and intermediate tubes to prevent
their melting and the deposition of carbon and salts on the inner tube. The outer tube carries the outer (sometimes referred
to as the plasma or coolant) gas, which is used to form and sustain the toroidal plasma. The tangential flow of the coolant
gas through the torch constricts the plasma and prevents the ICP from expanding to fill the outer tube, keeping the torch
from melting. An RF induction coil, also called the load coil, surrounds the torch and produces an oscillating magnetic field,
which in turn sets up an oscillating current in the ions and electrons produced from the argon. The impedance-matching
unit serves to efficiently couple the RF energy from the generator to the load coil. The unit can be either the active or
passive type. An active matching unit adjusts the impedance of the RF power by means of a capacitive network, whereas the
passive type adjusts the impedance directly through the generator circuitry. Within the load coil of the RF generator, the
energy transfer between the coil and the argon creates a self-sustaining plasma. Collisions of the ions and electrons liberated
from the argon ionize and excite the analyte atoms in the high-temperature plasma. The plasma operates at temperatures of
6,000 to 10,000 K, so most covalent bonds and analyte-to-analyte interactions have been eliminated.

**ICP–AES**

An inductively coupled plasma can use either an optical or a mass spectral detection system. In the former case, ICP–AES,
analyte detection is achieved at an emission wavelength of the analyte in question. Because of differences in technology, a
wide variety of ICP–AES systems are available, each with different capabilities, as well as different advantages and
disadvantages. Simultaneous-detection systems are capable of analyzing multiple elements at the same time, thereby
shortening analysis time and improving background detection and correction. Sequential systems move from one
wavelength to the next to perform analyses, and often provide a larger number of analytical lines from which to choose.
Array detectors, including charge-coupled devices and charge-injection devices, with detectors on a chip, make it possible to
combine the advantages of both simultaneous and sequential systems. These types of detection devices are used in the most
powerful spectrometers, providing rapid analysis and a wide selection of analytical lines.

The ICP can be viewed in either axial or radial (also called lateral) mode. The torch is usually positioned horizontally in
axially viewed plasmas and is viewed end on, whereas it is positioned vertically in radially viewed plasmas and is viewed from
the side. Axial viewing of the plasma can provide higher signal-to-noise ratios (better detection limits and precision);
however, it also incurs greater matrix and spectral interferences. Methods validated on an instrument with a radial
configuration will probably not be completely transferable to an instrument with an axial configuration, and vice versa.

Additionally, dual-view instrument systems are available, making it possible for the analyst to take advantage of either
torch configuration. The selection of the optimal torch configuration will depend on the sample matrix, analyte in question,
analytical wavelength(s) used, cost of instrumentation, required sensitivity, and type of instrumentation available in a given
laboratory.

Regardless of torch configuration or detector technology, ICP–AES is a technique that provides a qualitative and/or
quantitative measurement of the optical emission from excited atoms or ions at specific wavelengths. These measurements
are then used to determine the analyte concentration in a given sample. Upon excitation, an atom or atomic ion emits an
array of different frequencies of light that are characteristic of the distinct energy transition allowed for that element. The
intensity of the light is generally proportional to the analyte concentration. It is necessary to correct for the background
emission from the plasma. Sample concentration measurements are usually determined from a working curve of known
standards over the concentration range of interest. It is, however, also possible to perform a single-point calibration under certain circumstances, such as with limit tests, if the methodology has been validated for sufficient specificity, sensitivity, linearity, accuracy, precision, ruggedness, and robustness.

Because there are distinct transitions between atomic energy levels, and because the atoms in an ICP are rather dilute, emission lines have narrow bandwidths. However, because the emission spectra from the ICP contain many lines, and because “wings” of these lines overlap to produce a nearly continuous background on top of the continuum that arises from the recombination of argon ions with electrons, a high-resolution spectrometer is required in ICP–AES. The decision regarding which spectral line to measure should include an evaluation of potential spectral interferences. All atoms in a sample are excited simultaneously; however, the presence of multiple elements in some samples can lead to spectral overlap. Spectral interference can also be caused by background emission from the sample or plasma. Modern ICPs usually have background correction available, and a number of background correction techniques can be applied. Simple background correction typically involves measuring the background emission intensity at some point away from the main peak and subtracting this value from the total signal being measured. Mathematical modeling to subtract the interfering signal as a background correction can also be performed with certain types of ICP–AES spectrometers.

The selection of the analytical spectral line is critical to the success of an ICP–AES analysis, regardless of torch configuration or detector type. Though some wavelengths are preferred, the final choice must be made in the context of the sample matrix, the type of instrument being used, and the sensitivity required. Analysts might choose to start with the wavelengths recommended by the manufacturer of their particular instrument, and select alternative wavelengths based on manufacturer recommendations or published wavelength tables. Ultimately, the selection of analytical wavelengths should be demonstrated experimentally to provide sufficient specificity, sensitivity, linearity, accuracy, and precision of the analysis at hand.

Forward power, gas flow rates, viewing height, and torch position can all be optimized to provide the best signal. However, it must also be kept in mind that these same variables can influence matrix and spectral interferences.

In general, it is desirable to operate the ICP under robust conditions, which can be gauged on the basis of the MgII/MgI line pair at (280.270 nm/285.213 nm). If that ratio of intensities is above 6.0 in an aqueous solution, the ICP is said to be robust, and is less susceptible to matrix interferences. A ratio of about 10.0 is generally what is sought. Note that the term robust conditions is unrelated to robustness as applied to analytical method validation. Operation of an instrument with an MgII/MgI ratio greater than 6.0 is not mandated, but is being suggested as a means of optimizing instrument parameters in many circumstances.

The analyses of the Group I elements can be an exception to this strategy. When atomic ions are formed from elements in this group, they assume a noble gas electron configuration, with correspondingly high excitation energy. Because the first excited state of these ions is extremely high, few are excited, so emission intensity is correspondingly low. This situation can be improved by reducing the fractional ionization, which can in turn be achieved by using lower forward power settings in combination with adjusted viewing height or nebulizer gas flow, or by adding an ionization suppression agent to the samples and standards.

When organic solvents are used, it is often necessary to use a higher forward power setting, higher intermediate and outer gas flows, and a lower nebulizer gas flow than would be employed for aqueous solutions, as well as a reduction in the nebulizer gas flow. When using organic solvents, it may also be necessary to bleed small amounts of oxygen into the torch to prevent carbon buildup in the torch.

**Calibration:** The wavelength accuracy for ICP–AES detection must comply with the manufacturer’s applicable operating procedures. Because of the inherent differences among the types of instruments available, there is no general system suitability procedure that can be employed. Calibration routines recommended by the instrument manufacturer for a given ICP–AES instrument should be followed. These might include, but are not limited to, use of a multi-element wavelength calibration with a reference solution, internal mercury (Hg) wavelength calibration, and peak search. The analyst should perform system checks in accordance with the manufacturer’s recommendations.

**Standardization:** The instrument must be standardized for quantification at time of use. However, because ICP–AES is a technique generally considered to be linear over a range of 6–8 orders of magnitude, it is not always necessary to continually demonstrate linearity by the use of a standard curve composed of multiple standards. Once a method has been developed and is in routine use, it is possible to calibrate with a blank and a single standard. One-point standardizations are suitable for conducting limit tests on production materials and final products if the methodology has been rigorously validated for sufficient specificity, sensitivity, linearity, accuracy, precision, ruggedness, and robustness. The use of a single-point standardization is also acceptable for qualitative ICP–AES analyses, where the purpose of the experiment is to confirm the presence or absence of elements without the requirement of an accurate quantification.

An appropriate blank solution and standards that bracket the expected range of the sample concentrations should be assayed and the detector response plotted as a function of analyte concentration, as in the case where the concentration of a known component is being determined within a specified tolerance. However, it is not always possible to employ a bracketing standard when an analysis is performed at or near the detection limit. This lack of use of a bracketing standard is acceptable for analyses conducted to demonstrate the absence or removal of elements below a specified limit. The number and concentrations of standard solutions used should be based on the purpose of the quantification, the analyte in question, the desired sensitivity, and the sample matrix. Regression analysis of the standard plot should be employed to evaluate the linearity of detector response, and individual monographs may set criteria for the residual error of the regression line. Optimal, a correlation coefficient of NLT 0.99, or as indicated in the individual monograph, should be demonstrated for the working curve. Here, too, however, the nature of the sample matrix, the analyte(s), the desired sensitivity, and the type

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8 Harrison GR. Massachusetts Institute of Technology Wavelength Tables [also referred to as MIT Wavelength Tables]. Cambridge, MA: MIT Press; 1969.
of instrumentation available may dictate a correlation coefficient lower than 0.99. The analyst should use caution when proceeding with such an analysis, and should employ additional working standards.

To demonstrate the stability of the system's initial standardization, a solution used in the initial standard curve must be reassayed as a check standard at appropriate intervals throughout the analysis of the sample set. The reassayed standard should agree with its expected value to within ±10%, or as specified in an individual monograph, for single-element analyses when analytical wavelengths are 200–500 nm, or concentrations are >1 µg/mL. The reassayed standard should agree with its theoretical value to within ±20%, or as specified in an individual monograph, for multi-element analyses, when analytical wavelengths are <200 nm or >500 nm, or at concentrations of <1 µg/mL. In cases where an individual monograph provides different guidance regarding the reassayed check standard, the requirements of the monograph take precedence.

Procedure: Follow the procedure as directed in the individual monograph for the instrumental parameters. Because of differences in manufacturers’ equipment configurations, the manufacturer’s suggested default conditions may be used and modified as needed. The specification of definitive parameters in a monograph does not preclude the use of other suitable operating conditions, and adjustments of operating conditions may be necessary. Alternative conditions must be supported by suitable validation data, and the conditions in the monograph will take precedence for official purposes. Data collected from a single sample introduction are treated as a single result. This result might be the average of data collected from replicate sequential readings from a single solution introduction of the appropriate standard or sample solution. Sample concentrations are calculated versus the working curve generated by plotting the detector response versus the concentration of the analyte in the standard solutions. This calculation is often performed directly by the instrument.

ICP–MS

When an inductively coupled plasma uses a mass spectral detection system, the technique is referred to as inductively coupled plasma–mass spectrometry (ICP–MS). In this technique, analytes are detected directly at their atomic masses.

Because all masses must be charged to be detected in ICP–MS, the method relies on the ability of the plasma source to both atomize and ionize sample constituents. As is the case with ICP–AES, a wide variety of ICP–MS instrumentation systems are available.

The systems most commonly in use are quadrupole-based systems. Gaining in interest is time-of-flight ICP–MS. Although still not in widespread use, this approach may see greater use in the future. Additionally, high-resolution sector field instruments are available.

Regardless of instrument design or configuration, ICP–MS provides both a qualitative and a quantitative measurement of the components of the sample. Ions are generated from the analyte atoms by the plasma. The analyte ions are then extracted from the atmospheric-pressure plasma through a sampling cone into a lower-pressure zone, ordinarily held at a pressure near 1 Torr. In this extraction process, the sampled plasma gases, including the analyte species, form a supersonic beam, which dictates many of the properties of the resulting analyte ions. A skimmer cone, located behind the sampling cone, “skims” the supersonic beam of ions as they emerge from the sampling cone. Behind the skimmer cone is a lower-pressure zone, often held near a milliTorr. Lastly, the skinned ions pass a third-stage orifice to enter a zone held near a microTorr, where they encounter ion optics and are passed into the mass spectrometer. The mass spectrometer separates the ions according to their mass-to-charge (m/z) ratios. The ICP–MS has a mass range up to 240 atomic mass units (amu).

Depending on the equipment configuration, analyte adducts can form with diluents, with argon, or with their decomposition products. Also formed are oxides and multiply-charged analyte ions, which can increase the complexity of the resulting mass spectra. Interferences can be minimized by appropriate optimization of operational parameters, including gas flows (central, intermediate, and outer gas flow rates), sample-solution flow, RF power, extraction-lens voltage, etc., or by the use of collision or reaction cells, or cool plasma operation, if available on a given instrument. Unless a laboratory is generating or examining isotopes that do not naturally occur, a list of naturally occurring isotopes will provide the analyst with acceptable isotopes for analytical purposes. Isotopic patterns also serve as an aid to element identification and confirmation. Additionally, tables of commonly found interferences and polyatomic isotopic interferences and correction factors can be used.

ICP–MS generally offers considerably lower (better) detection limits than ICP–AES, largely because of the extremely low background that it generates. This ability is a major advantage of ICP–MS for determination of very low analyte concentrations or when elimination of matrix interferences is required. In the latter case, some interferences can be avoided simply by additional dilution of the sample solution. In some applications, analytes can be detected below the parts per trillion (ppt) level using ICP–MS. As a general rule, ICP–MS as a technique requires that samples contain significantly less total dissolved solids than does ICP–AES.

The selection of the analytical mass to use is critical to the success of an ICP–MS analysis, regardless of instrument design. Though some masses are often considered to be the primary ones, because of their high natural abundance, an alternative mass for a given element is often used to avoid spectral overlaps (isobaric interferences). Selection of an analytical mass must always be considered in the context of the sample matrix, the type of instrument being used, and the concentrations to be measured. Analysts might choose to start with masses recommended by the manufacturer of their particular instrument, and select alternate masses based on manufacturer’s recommendations or published tables of naturally occurring isotopes. Optimization of an ICP–MS method is also highly dependent on the plasma parameters and means of sample introduction. Forward power, gas flow rates, and torch position may all be optimized to provide the best signal. When organic solvents are used, it is often necessary to use a higher forward power setting and a lower nebulizer flow rate than would be used for aqueous solutions. Additionally, when organic solvents are used, it might be necessary to introduce small amounts of oxygen into the central or intermediate gas to prevent carbon buildup in the torch or on the sampler cone orifice. The use of a platinum-tipped sampling or skimmer cone may also be required in order to reduce cone degradation with some organic solvents.


Published on March 26, 2020
Calibration: The mass spectral accuracy for ICP–MS detection must be in accordance with the applicable operating procedures. Because of the inherent differences between the types of instruments available, there is no general system suitability procedure that can be employed. Analysts should refer to the tests recommended by the instrument manufacturer for a given ICP–MS instrument. These may include, but are not limited to, tuning on a reference mass or masses, peak search, and mass calibration. The analyst should perform system checks recommended by the instrument manufacturer.

Standardization: The instrument must be standardized for quantification at the time of use. Because the response (signal vs. concentration) of ICP–MS is generally considered to be linear over a range of 6–8 orders of magnitude, it is not always necessary to continually demonstrate linearity by the use of a working curve. Once a method has been developed and is in routine use, it is common practice to calibrate with a blank and a single standard. One-point standardizations are suitable for conducting limit tests on production materials and final products, provided that the methodology has been rigorously validated for sufficient specificity, sensitivity, linearity, accuracy, precision, ruggedness, and robustness. An appropriate blank solution and standards that bracket the expected range of the sample concentrations should be assayed and the detector response plotted as a function of analyte concentration. The number and concentration of standard solutions used should be based on the analyte in question, the expected concentrations, and the sample matrix, and should be left to the discretion of the analyst. Optimally, a correlation coefficient of NLT 0.99, or as indicated in the individual monograph, should be demonstrated for the working standard curve. Here, too, however, the nature of the sample matrix, the analyte, the desired sensitivity, and the type of instrumentation available might dictate a correlation coefficient lower than 0.99. The analyst should use caution when proceeding with such an analysis and should employ additional working standards.

To demonstrate the stability of the system since initial standardization, a solution used in the initial standard curve must be reassayed as a check standard at appropriate intervals throughout the analysis of the sample set. Appropriate intervals may be established as occurring after every fifth or tenth sample, or as deemed adequate by the analyst, on the basis of the analysis being performed. The reassayed standard should agree with its expected value to within ±10% for single-element analyses when analytical masses are free of interferences and when concentrations are >1 ng/mL. The reassayed standard should agree with its expected value to within ±20% for multi-element analyses, or when concentrations are <1 ng/mL. In cases where an individual monograph provides different guidance regarding the reassayed check standard, the requirements of the monograph take precedence.

The method of standard additions should be employed in situations where matrix interferences are expected or suspected. This method involves adding a known concentration of the analyte element to the sample solution at no fewer than two concentration levels. The instrument response is plotted against the concentration of the added analyte element, and a linear regression line is drawn through the data points. The absolute value of the x-intercept multiplied by any dilution factor is the concentration of the analyte in the sample.

Procedure: Follow the procedure as directed in the individual monograph for the detection mode and instrument parameters. The specification of definitive parameters in a monograph does not preclude the use of other suitable operating conditions, and adjustments of operating conditions may be necessary. Alternative conditions must be supported by suitable validation data, and the conditions in the monograph will take precedence for official purposes. Because of differences in manufacturers’ equipment configurations, the analyst may wish to begin with the manufacturer’s suggested default conditions and modify them as needed. Data collected from a single sample introduction are treated as a single result. Data collected from replicate sequential readings from a single introduction of the appropriate standard or sample solutions are averaged as a single result. Sample concentrations are calculated versus the working curve generated by plotting the detector response versus the concentration of the analyte in the standard solutions. With modern instruments, this calculation is often performed by the instrument.

GLOSSARY

Auxiliary gas: See Intermediate (or auxiliary) gas.
Axial viewing: A configuration of the plasma for AES in which the plasma is directed toward the spectrometer optical path, also called “end-on viewing.”
Central (or nebulizer) gas: One of three argon gas flows in an ICP torch. The central gas is used to help create a fine mist of the solution when solution nebulization is employed. This fine mist is then directed through the central tube of the torch and into the plasma.
Collision cell: A design feature of some ICP–MS instruments. Collision cells are used to reduce interferences from argon species or polyatomic ions and to facilitate the analysis of elements that might be affected by those interferences.
Cool plasma: Plasma conditions used for ICP–MS that result in a plasma that is cooler than that normally used for an analysis. This condition is achieved by using a lower forward power setting and higher central-gas flow rate, and is used to help reduce isotopic interferences caused by argon and some polyatomic ions.
Coolant gas: See Outer (or coolant or plasma) gas.
Forward power: The number of watts used to ignite and sustain the plasma during an analysis. Forward power requirements may vary, depending on sample matrix and analyte.
Intermediate (or auxiliary) gas: Gas used to “lift” the plasma off the surface of the torch, thereby preventing melting of the intermediate tube and the formation of carbon and salt deposits on the inner tube.
Internal standard: An element added to or present in the same concentration in blanks, standards, and samples to act as an intensity reference for the analysis. An internal standard should be used for ICP–AES work and must always be used for quantitative ICP–MS analyses.
m: The ion mass of interest
Lateral viewing: See Radial viewing.
Multiply-charged ions: Atoms that, when subjected to the high-ionization temperature of the ICP, can form doubly or triply charged ions (X++, X+++, etc.). When detected by MS, the apparent mass of these ions will be half or one-third that of the atomic mass.
Nebulizer: Used to form a consistent sample aerosol that mixes with the argon gas, which is subsequently sent into the ICP.
METHOD II (FOR LIQUIDS)

Unless otherwise directed, transfer the required weight of the sample onto a tared 75-mL to 100-mL platinum dish. Heat gently, using an Argand or Meker burner, until the sample ignites, then allow the sample to burn until it self-extinguishes. Cool, then wet the residue with 2 mL of concentrated sulfuric acid, and heat the sample over a low flame until dry. Ignite to constant weight in a muffle furnace at 800 ± 25° for 30 min, or longer if necessary for complete ignition, cool in a desiccator, and weigh.

Sieve Analysis of Granular Metal Powders (Based on ASTM Designation: B 214)\(^\text{13}\)

APPARATUS

Sieves: Use a set of standard sieves, ranging from 80-mesh to 325-mesh, conforming to the specifications in ASTM Designation: E 11 (Sieves for Testing Purposes).

Sieve shaker: Use a mechanically operated sieve shaker that imparts to the set of sieves a horizontal rotary motion of 270–300 rotations/min and a tapping action of 140–160 taps/min. The sieve shaker is fitted with a plug to receive the impact of the tapping device. The entire apparatus is rigidly mounted—bolted to a solid foundation, preferably of concrete. Preferably a time switch is provided to ensure the accuracy of test duration.

PROCEDURE

Assemble the sieves in consecutive order by opening size, with the coarsest sieve (80-mesh) at the top, and place a solid-collecting pan below the bottom sieve (325-mesh). Place 100.0 g of the test sample, W, on the top sieve, and close the sieve with a solid cover. Securely fasten the assembly to the sieve shaker, and operate the shaker for 15 min. Remove the most coarse sieve from the nest, gently tap its contents to one side, and pour the contents onto a tared, glazed paper. Using a soft brush, transfer onto the next finer sieve any material adhering to the bottom of the sieve and frame. Place the sieve just removed upside down on the paper containing the retained portion, and tap the sieve. Accurately weigh the paper and its contents, and record the net weight of the fraction, F, obtained. Repeat this process for each sieve in the nest and for the portion of the sample that has been collected in the bottom pan. Record the total of the fractions retained on the sieves as T and that portion collected in the pan as t. The combined total, S, of T + t is the amount of the sample, W, recovered in the test. Calculate the percent recovery:

\(^{13}\)Adapted from ASTM B214 Standard Test Method for Sieve Analysis of Metal Powders. The original ASTM method is available in its entirety from ASTM International, 100 Barr Harbor Drive, West Conshohocken, PA 19428. Phone: 610-832-9585, Fax: 610-832-9555, Email: service@astm.org, Website: www.astm.org.
Result = \( \frac{S}{W} \times 100 \)

If the percent recovery is less than 99.0%, check the condition of the sieves and for possible errors in weighing, and repeat the test. If the percent recovery is NLT 99.0%, calculate the percent retained on each sieve:

Result = \( \frac{F}{W} \times 100 \)

Calculate the percent through the smallest mesh sieve from the portion collected in the pan:

Result = \( \left( \frac{100 - t}{W} \right) \times 100 \)

### Sulfuric Acid Table

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Specific gravity determinations were made at 60°F, compared with water at 60°F. The values given above for aqueous sulfuric acid solutions were adopted as standard in 1904 by the Manufacturing Chemists’ Association of the United States.

From the specific gravities, the corresponding degrees Baumé were calculated by the following equation:

$$^\circ\text{Baumé} = 145 - \frac{145}{\text{sp. gr.}}$$

Baumé hydrometers for use with this table must be graduated by the above formula, which should always be printed on the scale. Acids stronger than 66°Bé should have their percentage compositions determined by chemical analysis.
Water-Insoluble Matter

SAMPLE PREPARATION

Add 5 g of sample (if a different amount of sample is specified in the individual monograph, use that amount) to 100 mL of water, and stir until the sample is dissolved.

PROCEDURE

Dry a membrane filter (cellulose nitrate, 0.45-µm porosity) at 110°C for 1 h, allow to cool in a desiccator, and weigh to the nearest 0.1 mg. Pass the Sample preparation through the dried membrane filter and wash with three successive 10-mL portions of water. Dry the membrane filter at 110°C for 1 h. Cool in a desiccator, and weigh the membrane filter to the nearest 0.1 mg. Calculate the insoluble matter as percentage.
APPENDIX III: CHEMICAL TESTS AND DETERMINATIONS

A. IDENTIFICATION TESTS

The identification tests described in section A of this Appendix are frequently referred to in the Food Chemicals Codex for the presumptive identification of FCC-grade chemicals taken from labeled containers. These tests are not intended to be applied to mixtures unless so specified.

**Acetate:** Acetic acid or acetates, when warmed with sulfuric acid and alcohol, form ethyl acetate, recognizable by its characteristic odor. With neutral solutions of acetates, ferric chloride TS produces a deep red color that is destroyed by the addition of a mineral acid.

**Aluminum:** Solutions of aluminum salts yield with 6 N ammonia a white, gelatinous precipitate that is insoluble in an excess of the 6 N ammonia. The same precipitate is produced by 1 N sodium hydroxide, but it dissolves in an excess of this reagent.

**Ammonium:** Ammonium salts are decomposed by 1 N sodium hydroxide with the evolution of ammonia, recognizable by its alkaline effect on moistened red litmus paper. The decomposition is accelerated by warming.

**Benzoate:** Neutral solutions of benzoates yield a salmon colored precipitate with ferric chloride TS. From moderately concentrated solutions of benzoate, 2 N sulfuric acid precipitates free benzoic acid, which is readily soluble in ether.

**Bicarbonate:** See Carbonate.

**Bisulfite:** See Sulfite.

**Bromide:** Free bromine is liberated from solutions of bromides upon the dropwise addition of chlorine TS. When shaken with chloroform, the bromine dissolves, coloring the chloroform red to red-brown. A yellow-white precipitate, which is insoluble in nitric acid and slightly soluble in 6 N ammonia, is produced when solutions of bromides are treated with silver nitrate TS.

**Calcium:** Insoluble oxalate salts are formed when solutions of calcium salts are treated in the following manner: using 2 drops of methyl red TS as the indicator, neutralize a 1:20 solution of a calcium salt with 6 N ammonia, then add 2.7 N hydrochloric acid, dropwise, until the solution is acid. A white precipitate of calcium oxalate forms upon the addition of ammonium oxalate TS. This precipitate is insoluble in acetic acid but dissolves in hydrochloric acid.

**Carbonate:** Carbonates and bicarbonates effervesce with acids, yielding a colorless gas that produces a white precipitate immediately when passed into calcium hydroxide TS. Cold solutions of soluble carbonates are colored red by phenolphthalein TS, whereas solutions of bicarbonates remain unchanged or are slightly changed.

**Chloride:** Solutions of chlorides yield with silver nitrate TS a white, curdy precipitate that is insoluble in nitric acid but soluble in a slight excess of 6 N ammonia.

**Citrate:** To 15 mL of pyridine add a few mg of a citrate salt, dissolved or suspended in 1 mL of water, and shake. Add 5 mL of acetic anhydride to this mixture, and shake. A light red color appears.

**Cobalt:** Solutions of cobalt salts (1:20) in 2.7 N hydrochloric acid yield a red precipitate when heated on a steam bath with an equal volume of a hot, freshly prepared 1:10 solution of 1-nitroso-2-naphthol in 9 N acetic acid. Solutions of cobalt salts yield a yellow precipitate when saturated with potassium chloride and treated with potassium nitrite and acetic acid.

**Copper:** When solutions of cupric compounds are acidified with hydrochloric acid, a red film of metallic copper is deposited on a bright untarnished surface of metallic iron. An excess of 6 N ammonia, added to a solution of a cupric salt, produces first a blue precipitate and then a deep blue colored solution. Solutions of cupric salts yield with potassium ferrocyanide TS a red-brown precipitate, insoluble in dilute acids.

**Hypophosphite:** Hypophosphites evolve spontaneously flammable phosphine when strongly heated. Solutions of hypophosphites yield a white precipitate with mercuric chloride TS. This precipitate becomes gray when an excess of hypophosphite is present. Hypophosphite solutions, acidified with sulfuric acid and warmed with copper sulfate TS, yield a red precipitate.

**Iodide:** Solutions of iodides, upon the dropwise addition of chlorine TS, liberate iodine, which colors the solution yellow to red. Chloroform is colored violet when shaken with this solution. The iodine thus liberated gives a blue color with starch TS. In solutions of iodides, silver nitrate TS produces a yellow, curdy precipitate that is insoluble in nitric acid and in 6 N ammonia.

**Iron:** Solutions of ferrous and ferric compounds yield a black precipitate with ammonium sulfide TS. This precipitate is dissolved by cold 2.7 N hydrochloric acid with the evolution of hydrogen sulfide.

**Ferric Salts:** Potassium ferrocyanide TS (10%) produces a dark blue precipitate in acid solutions of ferric salts. With an excess of 1 N sodium hydroxide, a red-brown precipitate is formed. Solutions of ferric salts produce with ammonium thiocyanate TS (1.0 N) a deep red color that is not destroyed by diluted mineral acids.

**Ferrous Salts:** Potassium ferrocyanide TS (10%) produces a dark blue precipitate in solutions of ferrous salts. This precipitate, which is insoluble in dilute hydrochloric acid, is decomposed by 1 N sodium hydroxide. Solutions of ferrous salts yield with 1 N sodium hydroxide a green-white precipitate, the color rapidly changing to green and then to brown when shaken.

**Lactate:** When solutions of lactates are acidified with sulfuric acid, potassium permanganate TS (0.1 N) is added, and the mixture is heated, acetaldehyde is evolved. This can be detected by allowing the vapor to come into contact with a filter paper that has been moistened with a freshly prepared mixture of equal volumes of 20% aqueous morpholine and sodium nitroferricyanide TS. A blue color is produced.
Magnesium: Solutions of magnesium salts in the presence of ammonium chloride yield no precipitate with ammonium carbonate TS, but a white crystalline precipitate, which is insoluble in 6 N ammonium hydroxide, is formed on the subsequent addition of sodium phosphate TS (6%).

Manganese: Solutions of manganous salts yield with ammonium sulfide TS a salmon colored precipitate that dissolves in acetic acid.

Nitrate: When a solution of a nitrate is mixed with an equal volume of sulfuric acid, the mixture cooled, and a solution of ferrous sulfate superimposed, a brown color is produced at the junction of the two liquids. Brown-red fumes are evolved when a nitrate is heated with sulfuric acid and metallic copper. Nitrates do not decolorize acidified potassium permanganate TS (0.1 N) (distinction from nitrites).

Nitrite: Nitrites yield brown-red fumes when treated with diluted mineral acids or acetic acid. A few drops of potassium iodide TS (15%) and a few drops of 2 N sulfuric acid added to a solution of nitrite liberate iodine, which colors starch TS blue.

Peroxide: Solutions of peroxides slightly acidified with sulfuric acid yield a deep blue color on the addition of potassium dichromate TS. On shaking the mixture with an equal volume of diethyl ether and allowing the liquids to separate, the blue color is transferred to the ether layer.

Phosphate: Neutral solutions of orthophosphates yield with silver nitrate TS (0.1 N) a yellow precipitate, which is soluble in 1.7 N nitric acid or in 6 N ammonium hydroxide. Acidified solutions of orthophosphates yield a yellow precipitate with ammonium molybdate TS, which is soluble in 6 N ammonium hydroxide.

Potassium: Potassium compounds impart a violet color to a nonluminous flame if not masked by the presence of small quantities of sodium. In neutral, concentrated or moderately concentrated solutions of potassium salts, sodium bitartrate TS (10%) slowly produces a white, crystalline precipitate that is soluble in 6 N ammonium hydroxide and in solutions of alkali hydroxides or carbonates. The precipitation may be accelerated by stirring or rubbing the inside of the test tube with a glass rod or by the addition of a small amount of glacial acetic acid or alcohol.

Sulfate: Solutions of sulfates yield with barium chloride TS a white precipitate that is insoluble in hydrochloric and nitric acids. Sulfates yield with lead acetate TS a white precipitate that is soluble in ammonium acetate solution. Hydrochloric acid produces no precipitate when added to solutions of sulfates (distinction from thiosulfates).

Sulfite: When treated with 2.7 N hydrochloric acid, sulfites and bisulfites yield sulfur dioxide, recognizable by its characteristic odor. This gas blackens filter paper moistened with mercuric nitrate TS.

Tartrate: When a few mg of a tartrate are added to a mixture of 15 mL of pyridine and 5 mL of acetic anhydride, an emerald green color is produced.

Thiosulfate: With hydrochloric acid, solutions of thiosulfates yield a white precipitate that soon turns yellow, liberating sulfur dioxide, recognizable by its odor. The addition of ferric chloride TS to solutions of thiosulfates produces a dark violet color that quickly disappears.

Zinc: Zinc salts, in the presence of sodium acetate, yield a white precipitate with hydrogen sulfide. This precipitate, which is insoluble in acetic acid, is dissolved by 2.7 N hydrochloric acid. A similar precipitate is produced by ammonium sulfide TS in neutral or alkaline solutions. Solutions of zinc salts yield with potassium ferrocyanide TS (10%) a white precipitate that is insoluble in 2.7 N hydrochloric acid.

B. LIMIT TESTS

ALUMINUM LIMIT TEST

[NOTE—The Standard solutions and Sample solution may be modified, if necessary, to obtain solutions of suitable concentrations adaptable to the linear or working range of the instrument.]

Nitric acid diluent: Dilute 40 mL of nitric acid with water to 1000 mL.

Standard aluminum solutions: Treat a quantity of aluminum wire with 6 N hydrochloric acid at 80° for a few min. Dissolve 100 mg of the treated wire in a mixture consisting of 10 mL of hydrochloric acid and 2 mL of nitric acid, by heating at 80° for about 30 min. Continue heating until the volume is reduced to about 4 mL. Cool to room temperature, and add 4 mL of water. Evaporate to about 2 mL by heating. Cool, and transfer this solution, with the aid of water, to a 100-mL volumetric flask, and dilute with water to volume (1 mg/mL aluminum). Transfer 10.0 mL of this solution to a second 100-mL volumetric flask, and dilute with water to volume (100 µg/mL aluminum). Transfer 1.0 mL of this solution to a third 100-mL volumetric flask, and dilute with water to volume (1 µg/mL aluminum). [NOTE—If more diluted Standard aluminum solutions are required, transfer 1.0-mL, 2.0-mL, and 4.0-mL portions of the 1 µg/mL Standard aluminum solution to separate 100-mL volumetric flasks, dilute with Nitric acid diluent to volume, and mix. These solutions contain 0.01 µg/mL, 0.02 µg/mL, and 0.04 µg/mL of aluminum, respectively.]

Sample solution: Transfer the amount of sample specified in the monograph to a plastic 100-mL volumetric flask. Add 50 mL of water, and sonicate for 30 min. Add 4 mL of nitric acid, and dilute with water to volume.

Procedure: Determine the absorbances of the Standard aluminum solutions and the Sample solution at the aluminum emission line at 309.3 nm with a suitable atomic absorption spectrophotometer equipped with an aluminum hollow-cathode lamp and a flameless electrically heated furnace, using the Nitric acid diluent as the blank. Plot the absorbances of the Standard solutions versus the concentration of aluminum, in µg/mL, drawing a straight line best fitting the three points. From the graph so obtained, determine the concentration, in µg/mL, of aluminum in the Sample solution. Calculate the amount of aluminum in the sample taken, in µg/g.
Result = \( \frac{C_A}{C_S} \)

in which \( C_A \) is the concentration of aluminum in the *Sample solution*, in µg/mL, obtained from the standard curve; and \( C_S \) is the concentration of the *Sample solution*, in g/mL.

**ARSENIC LIMIT TEST**

*Silver Diethylthiocarbamate Colorimetric Method*

[NOTE—All reagents used in this test should be very low in arsenic content.]

**Apparatus:** Use the general apparatus shown in *Figure 1* unless otherwise specified in an individual monograph. It consists of a 125-mL arsine generator flask (a) fitted with a scrubber unit (c) and an absorber tube (e), with a 24/40 standard-taper joint (b) and a ball-and-socket joint (d), secured with a No. 12 clamp, connecting the units. The tubing between d and e and between d and c is a capillary having an id of 2 mm and an od of 8 mm. Alternatively, an apparatus embodying the principle of the general assembly described and illustrated may be used.

![Figure 1. General Apparatus for Arsenic Limit Test (Courtesy of the Fisher Scientific Co., Pittsburgh, PA.)](image)

[NOTE—The special assemblies shown in *Figures 2, 3, and 4* are to be used only when specified in certain monographs.]  

![Figure 2. Modified Bethge Apparatus for the Distillation of Arsenic Tribromide](image)
Standard arsenic solution: Accurately weigh 132.0 mg of arsenic trioxide that has been previously dried at 105° for 1 h, and dissolve it in 5 mL of a 1:5 sodium hydroxide solution. Neutralize the solution with 2 N sulfuric acid, add 10 mL in excess, and dilute with recently boiled water to 1000.0 mL. Transfer 10.0 mL of this solution into a 1000-mL volumetric flask, add 10 mL of 2 N sulfuric acid, dilute with recently boiled water to volume, and mix. Use this final solution, which contains 1 μg of arsenic in each mL, within 3 days.

Silver diethyldithiocarbamate solution: Dissolve 1 g of ACS reagent-grade silver diethyldithiocarbamate in 200 mL of recently distilled pyridine. Store this solution in a light-resistant container and use within 1 month.

Stannous chloride solution: Dissolve 40 g of stannous chloride dihydrate (SnCl₂·2H₂O) in 100 mL of hydrochloric acid. Store the solution in glass containers and use within 3 months.

Lead acetate-impregnated cotton: Soak cotton in a saturated solution of lead acetate trihydrate, squeeze out the excess solution, and dry in a vacuum at room temperature.

Sample solution: Use directly as the Sample solution in the Procedure the solution obtained by treating the sample as directed in an individual monograph. Prepare Sample solutions of organic compounds in the generator flask (a), unless otherwise directed, according to the following general procedure:

[CAUTION—Some substances may react unexpectedly with explosive violence when digested with hydrogen peroxide. Use appropriate safety precautions at all times.]

[Note—If halogen-containing compounds are present, use a lower temperature while heating the sample with sulfuric acid; do not boil the mixture; and add the peroxide, with caution, before charring begins to prevent loss of trivalent arsenic.]

Transfer 1.0 g of the sample into the generator flask, add 5 mL of sulfuric acid and a few glass beads, and digest at a temperature not exceeding 120° until charring begins, preferably using a hot plate in a fume hood. (Additional sulfuric acid may be necessary to completely wet some samples, but the total volume added should not exceed about 10 mL.) After the acid has initially decomposed the sample, cautiously add, dropwise, hydrogen peroxide (30%), allowing the
reaction to subside and reheating the sample between drops. Add the first few drops very slowly with sufficient mixing to prevent a rapid reaction, and discontinue heating if foaming becomes excessive. Swirl the solution in the flask to prevent unreacted substance from caking on the walls or bottom of the flask during digestion.

[Note—Maintain oxidizing conditions at all times during the digestion by adding small quantities of the peroxide whenever the mixture turns brown or darkens.]

Continue the digestion until the organic matter is destroyed, gradually raising the temperature of the hot plate to 250° to 300° until fumes of sulfur trioxide are copiously evolved and the solution becomes colorless or retains only a light straw color. Cool, cautiously add 10 mL of water, heat again to strong fuming, and cool. Cautiously add 10 mL of water, mix, wash the sides of the flask with a few mL of water, and dilute to 35 mL.

**Procedure:** If the *Sample solution* was not prepared in the generator flask, transfer to the flask a volume of the solution, prepared as directed, equivalent to 1.0 g of the substance being tested, and add water to make 35 mL. Add 20 mL of 1:5 sulfuric acid, 2 mL of *potassium iodide TS*, 0.5 mL of *Stannous chloride solution*, and 1 mL of isopropyl alcohol, and mix. Allow the mixture to stand for 30 min at room temperature. Pack the scrubber unit (c) with two plugs of *Lead acetate-impregnated cotton*, leaving a small air space between the two plugs, lubricate joints b and d with stopcock grease, if necessary, and connect the scrubber unit with the absorber tube (e). Transfer 3.0 mL of *Silver diethyldithiocarbamate solution* to the absorber tube, add 3.0 g of granular zinc (20-mesh) to the mixture in the flask, and immediately insert the standard-taper joint (b) into the flask. Allow the evolution of hydrogen and color development to proceed at room temperature (25 ± 3°) for 45 min, swirling the flask gently at 10-min intervals. Disconnect the absorber tube from the generator and scrubber units, and transfer the *Silver diethyldithiocarbamate solution* to a 1-cm absorption cell. Determine the absorbance at the wavelength of maximum absorption between 535 nm and 540 nm, with a suitable spectrophotometer or colorimeter, using *Silver diethyldithiocarbamate solution* as the blank. The absorbance due to any red color from the solution of the sample does not exceed that produced by 3.0 mL of *Standard arsenic solution* (3 µg As) when treated in the same manner and under the same conditions as the sample. The room temperature during the generation of arsine from the standard should be held to within ±2° of that observed during the determination of the sample.

**Interferences:** Metals or salts of metals such as chromium, cobalt, copper, mercury, molybdenum, nickel, palladium, and silver may interfere with the evolution of arsine. Antimony, which forms stibine, is the only metal likely to produce a positive interference in the color development with the silver diethyldithiocarbamate. Stibine forms a red color with silver diethyldithiocarbamate that has a maximum absorbance at 510 nm, but at 535–540 nm, the absorbance of the antimony complex is so diminished that the results of the determination would not be altered significantly.

**CADMIUM LIMIT TEST**

**Spectrophotometer:** Use any suitable atomic absorption spectrophotometer equipped with a Boling-type burner, an air–acetylene flame, and a hollow-cathode cadmium lamp. The instrument should be capable of operating within the sensitivity necessary for the determination.

**Standard solution:** Transfer 100 mg of cadmium chloride crystals (CdCl₂·2½H₂O), accurately weighed, into a 1000-mL volumetric flask, dissolve in and dilute with water to volume, and mix. Pipet 25 mL of this solution into a 100-mL volumetric flask, add 1 mL of hydrochloric acid, dilute with water to volume, and mix. Each mL contains 12.5 µg of cadmium.

**Sample solution:** Transfer 10 g of sample, accurately weighed, into a 50-mL volumetric flask, dissolve in and dilute with water to volume, and mix.

**Test solutions:** Transfer 5.0 mL of the *Sample solution* into each of five separate 25-mL volumetric flasks. Dilute the contents of Flask 1 with water to volume, and mix. Add 1.00 mL, 2.00 mL, 3.00 mL, and 4.00 mL of *Standard solution* to Flasks 2, 3, 4, and 5, respectively; then dilute each flask with water to volume; and mix. The *Test solutions* contain, respectively, 0 µg/mL, 0.5 µg/mL, 1.0 µg/mL, 1.5 µg/mL, and 2.0 µg/mL of cadmium.

**Procedure:** If the *Sample solution* was not prepared in the generator flask, transfer to the flask a volume of the sample. The room temperature during the generation of arsine from the standard should be held to within ±2° of that observed during the determination of the sample.

**Interferences:** Metals or salts of metals such as chromium, cobalt, copper, mercury, molybdenum, nickel, palladium, and silver may interfere with the evolution of arsine. Antimony, which forms stibine, is the only metal likely to produce a positive interference in the color development with the silver diethyldithiocarbamate. Stibine forms a red color with silver diethyldithiocarbamate that has a maximum absorbance at 510 nm, but at 535–540 nm, the absorbance of the antimony complex is so diminished that the results of the determination would not be altered significantly.

**CHLORIDE AND SULFATE LIMIT TESTS**

Where limits for chloride and sulfate are specified in the individual monograph, compare the *Sample solution* and control in appropriate glass cylinders of the same dimensions and matched as closely as practicable with respect to their optical characteristics.

If the solution is not perfectly clear after acidification, pass it through filter paper that has been washed free of chloride and sulfate. Add identical quantities of the precipitant (*silver nitrate TS* or *barium chloride TS*) in rapid succession to both the *Sample solution* and the control solution.

Experience has shown that visual turbidimetric comparisons are best made between solutions containing from 10 to 20 µg of chloride (Cl) ion or from 200 to 400 µg of sulfate (SO₄) ion in 50 mL. Weights of samples are specified on this basis in the individual monographs in which these limits are included.

• **CHLORIDE LIMIT TEST**

**Standard chloride solution:** Dissolve 165 mg of sodium chloride in water and dilute to 100.0 mL. Transfer 10.0 mL of this solution into a 1000-mL volumetric flask, dilute with water to volume, and mix. Each mL of the final solution contains 10 µg of chloride (Cl) ion.

**Procedure:** Unless otherwise directed, dissolve the specified amount of the test substance in 30–40 mL of water; neutralize to litmus external indicator with nitric acid, if necessary; and add 1 mL in excess. Add 1 mL of *silver nitrate TS* to the clear solution or filtrate, dilute with water to 50 mL, mix, and allow to stand for 5 min protected from direct

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sunlight. Compare the turbidity, if any, with that produced similarly in a control solution containing the required volume of Standard chloride solution and the quantities of the reagents used for the sample.

**Sulfate Limit Test**

**Standard sulfate solution:** Dissolve 148 mg of anhydrous sodium sulfate in water, and dilute to 100.0 mL. Transfer 10.0 mL of this solution to a 1000-mL volumetric flask, dilute with water to volume, and mix. Each mL of the final solution contains 10 µg of sulfate (SO₄²⁻).

**Procedure:** Unless otherwise directed, dissolve the specified amount of the test substance in 30–40 mL of water; neutralize to litmus external indicator with hydrochloric acid, if necessary; then add 1 mL of 2.7 N hydrochloric acid. Add 3 mL of barium chloride TS to the clear solution or filtrate, dilute with water to 50 mL, and mix. After 10 min compare the turbidity, if any, with that produced in a solution containing the required volume of Standard sulfate solution and the quantities of the reagents used for the sample.

**Copper Limit Test**

**Flame Atomic Absorption Spectrometric Method**

[Note—Soak all glassware in 10% nitric acid for over 24 h, then rinse them thoroughly with water, followed by deionized water. Dry all glassware before usage.]

**Dilution solution:** Dilute 0.5 mL concentrated nitric acid with water to 100 mL.

**Copper stock solution (1000 µg/mL):** Dissolve 1000.0 mg of copper (Cu, 99.99%) in 40% nitric acid (total amount NMT 37 mL) in a 1000-mL volumetric flask, and dilute with water to volume.

**Diluted standard copper solutions:** Transfer 10 mL of Copper stock solution into a 100-mL volumetric flask, and dilute with Dilution solution to volume. Transfer 10 mL of the obtained solution into a 100-mL volumetric flask, and dilute with Dilution solution to volume. The obtained solution is further diluted with the same method to create a working solution with a copper concentration of 1 µg/mL. Then, further dilute the working solution (again, with Dilution solution) to create “Diluted standard copper solutions” containing 0.10 µg/mL, 0.20 µg/mL, 0.40 µg/mL, 0.60 µg/mL, 0.80 µg/mL, and 1.00 µg/mL of copper.

**Sample preparation:** Transfer 2.0 g of sample to a quartz or porcelain crucible, add 5 mL concentrated nitric acid, and let the mixture stand for 30 min. Heat the mixture to dry using soft fire, then continue heating to carbonize the mixture. Transfer the carbonized material to a muffle furnace, and continue the carbonization at 500° ± 25° for 1 h. Remove the carbonized sample from the furnace, and cool at room temperature. Add 1 mL concentrated nitric acid to wet the ash in the crucible, and then evaporate it carefully until dry. Carbonize the material in a muffle furnace again at 500° for 30 min, then remove it from the furnace and allow it to cool to room temperature. To the ash obtained, add 1 mL of 20% nitric acid, transfer the solution to a 10-mL volumetric flask, repeat it 4 times, and then dilute with water to volume.

**Blank preparation:** Prepare as directed in the Sample preparation, replacing the sample with the same amount of concentrated nitric acid.

**Procedure:** Determine the absorbance of the Blank preparation, the Diluted standard copper solutions, and the Sample preparation at the copper emission line of 324.8 nm, using a slit-width of 0.5 nm. Use a suitable atomic absorption spectrophotometer equipped with a copper electrodeless discharge lamp (EDL), an air–acetylene flame, and a 4-in burner head. Use water as the blank.

**Calculations:** Determine the corrected absorbance values by subtracting the Blank preparation absorbance from each of the Diluted standard copper solutions and from the Sample preparation absorbance. Prepare a standard curve by plotting the corrected Diluted standard copper solutions absorbance values versus their corresponding concentrations expressed as µg/mL. Determine the copper concentration in the Sample preparation by reference to the calibration curve. Calculate the quantity of copper, in mg/kg, in the sample taken:

\[
\text{Result} = \frac{10C}{W_s}
\]

\[
C = \text{concentration of copper from the Standard curve (µg/mL)}
\]

\[
W_s = \text{weight of the sample taken (g)}
\]

**Atomic Absorption Spectrophotometric Graphite Furnace Method**

[Note—Soak all glassware in 10% nitric acid for over 24 h, then rinse them thoroughly with water, followed by deionized water. Dry all glassware before usage.]

**Apparatus:** Use a suitable graphite furnace atomic absorption spectrophotometer set at 324.8 nm and equipped with an autosampler, pyrolytically coated graphite tubes, solid pyrolytic graphite platforms, and an adequate means of background correction. Zeeman effect or Smith-Hieftje background correction is preferred, but deuterium arc background correction should be acceptable. (This method was developed on a Perkin-Elmer Model ZS100, 0.7-nm slit, HGA-600 furnace, AS-60 autosampler with Zeeman background correction.) If the instrument does not have a well-defined calibration function, a separate calculator or computer is required for linear least squares, nonlinear, or quadratic calibrations. Set up the instrument according to the manufacturer’s specifications with consideration of current good GFAAS practices—addressing such factors as line voltage, cooling water temperature, graphite part specifications, and furnace temperature. If an optical pyrometer or thermocouple is not available to check the furnace constant temperature, calibration, and observe the furnace emission through the sample introduction port while increasing the furnace temperature. A characteristic cherry red glow should begin to appear at 800°. If it glows at a lower temperature, then the furnace is hotter, and temperatures must be adjusted downward accordingly.
Use acid-cleaned [in a mixture of 5% sub-boiling, distilled nitric acid and 5% sub-boiling, distilled hydrochloric acid made up in deionized, distilled water (18 megohm), and thoroughly rinsed with deionized, distilled water (18 megohm)] autosampler cups (PE B008–7600 Teflon, or equivalent) to avoid contamination. Use micropipets with disposable tips free of contamination for dilution. Ensure accuracy and precision of micropipets and tips by dispensing and weighing 5–10 replicate portions of water onto a microbalance. Use acid-cleaned volumetric glassware to prepare standards and dilute samples to a final volume. For digestion, use acid-cleaned, high-density polyethylene tubes, polypropylene tubes, Teflon tubes, or quartz tubes. Store final diluted samples in plastic tubes.

Air ashing: The furnace controller must be able to handle two gas flows to facilitate air ashing. Oxygen ashing is used to avoid build up of residue during the char step. Argon is used as the purge gas for the furnace for all steps but the char. Breathing quality air can be used as the alternate gas for the air ashing.

Dilution solution: Dilute 0.5 mL concentrated nitric acid to 100 mL with water

Copper stock solution (1000 µg/mL): Dissolve 1000.0 mg of copper (Cu, 99.99%) in 40% nitric acid (total amount NMT 37 mL) in a 1000-mL volumetric flask, and dilute with water to volume.

Diluted standard copper solutions: Transfer 10 mL of Copper stock solution into a 100-mL volumetric flask, and dilute with Dilution solution to volume. Transfer 10 mL of the obtained solution into a 100-mL volumetric flask, and dilute with Dilution solution to volume. The obtained solution is further diluted with the same method to create a working solution with a copper concentration of 0.1 µg/mL. Then further dilute the working solution (again, with Dilution solution) to create “Diluted standard copper solutions” containing 0.01 µg/mL, 0.02 µg/mL, 0.04 µg/mL, 0.06 µg/mL, 0.08 µg/mL, and 0.10 µg/mL of copper.

Sample preparation: Transfer 2.0 g of sample to a quartz or porcelain crucible, add 5 mL concentrated nitric acid, and let the mixture stand for 30 min. Heat the mixture carefully until dry, then continue heating to carbonize the mixture. Transfer the carbonized material to a muffle furnace, and continue the carbonization at 500° ± 25° for 1 h. Remove the carbonized sample from the furnace, and cool at room temperature. Add 1 mL concentrated nitric acid to wet the ash in the crucible, and then evaporate it carefully until dry. Carbonize the material in a muffle furnace again at 500° for 30 min, then remove it from the furnace and allow it to cool to room temperature. To the ash obtained, add 1 mL of 20% nitric acid, transfer the solution to a 10-mL volumetric flask, repeat it 4 times, and then dilute with water to volume.

Blank preparation: Prepare as directed in the Sample preparation, replacing the sample with the same amount of concentrated nitric acid.

Procedure: Inject 10–20 µL Sample preparation, Blank preparation, and Diluted standard copper solutions into a graphite furnace atomizer. The furnace program is as follows: (1) Dry at 90°, using a 20-s hold and a 1500-mL/min argon flow (or other inert gas); (2) char the sample at 800°, using a 20-s hold and a constant speed of air flow; [NOTE—Air ashing is a critical step to avoid build up of residue during the char step.] (3) cool down, and purge the air from the furnace for 60 s, using a temperature set to 20° and a 1500-mL/min argon flow (or other inert gas); (4) atomize at 2300°, using a 4-s hold with the argon flow (or other inert gas) stopped. Determine the absorbance at the copper emission line of 324.8 nm, using a slit-width of 0.5 nm.

[NOTE—If the test has interference from sodium chloride or other materials, either dilute the Sample preparation with 1 mg/mL ammonium nitrate or ammonium dihydrogen phosphate before injection, or add the same quantity of ammonium nitrate or ammonium dihydrogen as a chemical modifier after injection (into graphite furnace).]

Calculations: Determine the corrected absorbance values by subtracting the Blank preparation absorbance from each of the Diluted standard copper solutions and from the Sample preparation absorbance. Prepare a Standard curve by plotting the corrected Diluted standard copper solutions absorbance values versus their corresponding concentrations expressed as µg/mL. Determine the copper concentration in the Sample preparation by reference to the calibration curve. Calculate the quantity of copper, in mg/kg, in the sample taken:

\[ \text{Result} = \frac{10C}{W_s} \]

\[ C = \text{concentration of copper from the standard curve (µg/mL)} \]

\[ W_s = \text{weight of the sample taken (g)} \]

1.4-DIOXANE LIMIT TEST

Vacuum distillation apparatus: Assemble a closed-system vacuum distillation apparatus employing glass vacuum stopcocks (A, B, and C), as shown in Figure 3.
The concentrator tube (D) is made of borosilicate or quartz (not flint) glass, graduated precisely enough to measure the 0.9 mL or more of distillate and marked so that the analyst can accurately dilute to 2.0 mL (available as Chromaflex concentrator tube, Kontes Glass Co., Vineland, NJ, Catalog No. K42560-0000).

**Standard preparation:** Prepare a solution of 1,4-dioxane in water containing 100 µg/mL. Keep the solution refrigerated, and prepare fresh weekly.

**Sample preparation:** Transfer 20 g of the sample, accurately weighed, into a 50-mL round-bottom flask (E) having a 24/40 ground-glass neck. Semisolid or waxy samples should be liquefied by heating on a steam bath before making the transfer. Add 2.0 mL of water to the flask for crystalline samples, and 1.0 mL for liquid, semisolid, or waxy samples. Place a small Teflon-covered stirring bar in the flask, stopper, and stir to mix. Immerse the flask in an ice bath, and chill for about 1 min.

Wrap heating tape around the tube connecting the Chromaflex tube (D) and the round-bottom flask (E), and apply about 10 V to the tape. Apply a light coating of high-vacuum silicone grease to the ground-glass joints, and connect the Chromaflex tube to the 10/30 joint and the round-bottom flask to the 24/40 joint. Immerse the vacuum trap in a Dewar flask filled with liquid nitrogen, close stopcocks A and B, open stopcock C, and begin evacuating the system with a vacuum pump. Prepare a slush bath from powdered dry ice and methanol, and raise the bath to the neck of the round-bottom flask. After freezing the contents of the flask for about 10 min, and when the vacuum system is operating at 0.05 mm pressure or lower, open stopcock A for 20 s, and then close it. Remove the slush bath, and allow the flask to warm in air for about 1 min. Immerse the flask in a water bath at 20\(^\circ\)–25\(^\circ\), and after about 5 min warm the water in the bath to 35\(^\circ\)–40\(^\circ\) (sufficient to liquefy most samples) while stirring slowly but constantly with the magnetic bar. Cool the water in the bath by adding ice, and chill for about 2 min. Replace the water bath with the slush bath, freeze the contents of the flask for about 10 min, then open stopcock A for 20 s, and close it. Remove the slush bath, and repeat the heating steps as before, this time reaching a final temperature of 45\(^\circ\)–50\(^\circ\) or a temperature necessary to melt the sample completely. If there is any condensation in the tube connecting the round-bottom flask to the Chromaflex tube, slowly increase the voltage to the heating tape and heat until condensation disappears.

Stir with the magnetic stirrer throughout the following steps: very slowly immerse the Chromaflex tube in the Dewar flask containing liquid nitrogen. **CAUTION—**When there is liquid distillate in the Chromaflex tube, the tube must be immersed in the nitrogen very slowly, or the tube will break.

Water will begin to distill into the tube. As ice forms in the tube, raise the Dewar flask to keep the liquid nitrogen level only slightly below the level of ice in the tube. When water begins to freeze in the neck of the 10/30 joint, or when liquid nitrogen reaches the 2.0-mL graduation mark on the Chromaflex tube, remove the Dewar flask and let the ice melt without heating. After the ice has melted, check the volume of water that has distilled, and repeat the sequence of chilling and thawing until at least 0.9 mL of water has been collected. Freeze the tube once again for about 2 min, and release the vacuum first by opening stopcock B, followed by stopcock A. Remove the Chromaflex tube from the apparatus, close it with a greased stopper, and let the ice melt without heating. Mix the contents of the tube by swirling, note the volume of distillate, and dilute with water to 2.0 mL, if necessary. Use this Sample preparation as directed under Chromatography.

**Chromatography:** (See Chromatography, Appendix II.) Use a gas chromatograph equipped with a flame-ionization detector. Under typical conditions, the instrument contains a 4-mm (id) \times 6-ft glass column, or equivalent, packed with 80-100- or 100-120-mesh Chromosorb 104, or equivalent. The column is maintained isothermally at about 140\(^\circ\), the injection port at 200\(^\circ\), and the detector at 250\(^\circ\). Nitrogen is the carrier gas, flowing at a rate of about 35 mL/min. Install an oxygen scrubber between the carrier gas line and the column. The column should be conditioned for about 72 h at 250\(^\circ\) with 30–40 mL/min carrier flow.

**Note—**Chromosorb 104 is oxygen sensitive. Both new and used columns should be flushed with carrier gas for 30–60 min before heating each time they are installed in the gas chromatograph.

Inject a volume of the Standard preparation, accurately measured, to give about 20% of maximum recorder response. Where possible, keep the injection volume in the range of 2–4 µL, and use the solvent-flush technique to minimize errors associated with injection volumes. In the same manner, inject an identical volume of the Sample preparation. The height of the peak produced by the Sample preparation does not exceed that produced by the Standard preparation.\(^1\)

---

\(^1\) If the sample fails the test because of known or suspected interference, another aliquot may be run on a 6-ft \times 2-mm (id) column, or equivalent, of 0.2% Carbowax 1500 on Carbopak C, operating at 100\(^\circ\) isothermal, with 20 mL/min of helium carrier flow. Under these conditions, the 1,4-dioxane elutes in about 4 min.
FLUORIDE LIMIT TEST

**Method I (Thorium Nitrate Colorimetric Method)**

Use this method unless otherwise directed in the individual monograph.

**[CAUTION]**—When applying this test to organic compounds, rigidly control at all times the temperature at which the distillation is conducted to the recommended range of 135°–140° to avoid the possibility of explosion.

**[NOTE]**—To minimize the distillation blank resulting from fluoride leached from the glassware, treat the distillation apparatus as follows: treat the glassware with hot 10% sodium hydroxide solution, followed by flushing with tap water and rinsing with distilled water. At least once daily, treat in addition by boiling down 15–20 mL of 1:2 sulfuric acid until the still is filled with fumes; cool, pour off the acid, treat again with 10% sodium hydroxide solution, and rinse thoroughly. For further details, see AOAC method 944.08.

Unless otherwise directed, place a 5.0-g sample and 30 mL of water in a 125-mL Pyrex distillation flask with a side arm and trap. The flask is connected with a condenser and carries a thermometer and a capillary tube, both of which must extend into the liquid. Slowly add, with continuous stirring, 10 mL of 70% perchloric acid, and then add 2 or 3 drops of a 1:2 solution of silver nitrate and a few glass beads. Connect a small dropping funnel or a steam generator to the capillary tube. Support the flask on a flame-resistant mat or shielding board, with a hole that exposes about one-third of the flask to the low, “clean” flame of a Bunsen burner.

**[NOTE]**—The shielding is essential to prevent the walls of the flask from overheating above the level of its liquid contents.

Distill until the temperature reaches 135°. Add water from the funnel or introduce steam through the capillary, maintaining the temperature between 135° and 140° at all times. Continue the distillation until 100 mL of distillate has been collected. After the 100-mL portion (Distillate A) is collected, collect an additional 50-mL portion of distillate (Distillate B) to ensure that all of the fluorine has been volatilized.

Place 50 mL of Distillate A in a 50-mL Nessler tube. In another, similar Nessler tube, place 50 mL of water distilled through the apparatus as a control. Add to each tube 0.1 mL of a filtered 1:1000 solution of sodium alizarin sulfonate and 1 mL of a freshly prepared 1:4000 solution of hydroxylamine hydrochloride, and mix well. Add, dropwise and with stirring, either 1 N or 0.05 N sodium hydroxide, depending on the expected volume of volatile acid distilling over, to the tube containing the distillate until its color just matches that of the control, which is faintly pink. Then add to each tube 1.0 mL of 0.1 N hydrochloric acid, and mix well. From a buret, graduated in 0.05 mL, add slowly to the tube containing the distillate enough of a 1:4000 solution of thorium nitrate so that, after mixing, the color of the liquid just changes to a faint pink. Note the volume of the solution added, then add exactly the same volume to the control, and mix. Now add to the control solution sodium fluoride TS [10 µg of fluoride (F) per mL] from a buret to make the color of the two tubes match after dilution to the same volume. Mix well, and allow all air bubbles to escape before making the final color comparison. Check the endpoint by adding 1 or 2 drops of sodium fluoride TS to the control. A distinct change in color should take place. Note the volume of sodium fluoride TS added.

Dilute Distillate B to 100 mL, and mix well. Place 50 mL of this solution in a 50-mL Nessler tube, and follow the procedure used for Distillate A. The total volume of sodium fluoride TS required for the solutions from both Distillate A and Distillate B should not exceed 2.5 mL.

**Method II (Ion-Selective Electrode Method A)**

**Buffer solution**: Dissolve 36 g of cyclohexylenedinitrilotetraacetic acid (CDTA) in sufficient 1 N sodium hydroxide to make 200 mL. Transfer 20 mL of this solution (equivalent to 4 g of disodium CDTA) into a 1000-mL beaker containing 500 mL of water, 57 mL of glacial acetic acid, and 58 g of sodium chloride, and stir to dissolve. Adjust the pH of the solution to 5.0–5.5 by the addition of sodium hydroxide, then cool to room temperature, dilute with water to 1000 mL, and mix.

**Fluoride standard**: Transfer 2.2108 g of sodium fluoride, previously dried at 200° for 4 h, into a 1000-mL volumetric flask, and dilute with water to volume. The resulting solution contains 100 µg of fluoride (F) ion per mL.

**Fluoride standard solution**: Transfer 1 mL of the Fluoride standard to a 10-mL volumetric flask, and dilute with water to volume. The resulting solution contains 1000 µg of fluoride per mL.

**Electrode calibration**: Pipet 50 mL of the Buffer solution into a plastic beaker. Place the fluoride ion and reference electrodes (or a combination fluoride electrode) into the plastic beaker, and stir. At 5-min intervals, add 100 µL and 1000 µL of the Fluoride standard and read the potential, in mV, after each addition. The difference between the two readings is the slope of the fluoride electrode and should typically be in the range of 54–60 mV at 25° for the standards in the Buffer solution. If the difference in potential is not within this range, check, and, if necessary, replace the electrode, instrument, or solutions. Alternatively, the electrode calibration should be performed according to the manufacturer’s instructions and should comply with the manufacturer’s calibration range at 25°. If the difference in potential is not within this range, evaluate the system and equipment as necessary.

**Procedure**: Unless otherwise directed in the individual monograph, transfer 8.0 g of sample and 20 mL of water into a 250-mL distilling flask, cautiously add 20 mL of perchloric acid, and then add 2 or 3 drops of a 1:2 solution of silver nitrate and a few glass beads.

**[CAUTION]**—Handle perchloric acid in an appropriate fume hood.

Following the directions, and observing the Caution and Notes, as given under Method I, distill the solution until 200 mL of distillate has been collected.

Transfer a 25.0-mL aliquot of the distillate into a 250-mL plastic beaker, and dilute with the Buffer solution to 100 mL. Place the fluoride ion and reference electrodes (or a combination fluoride electrode) of a suitable ion-selective electrode apparatus in the solution. Allow sufficient time for equilibration (about 20 min), stirring constantly during the equilibration period and throughout the remainder of the procedure, and record the initial equilibrated...
Fluoride limit solutions (for a 1-g sample)

Buffer solution B:
Dissolve 150 g of sodium citrate dihydrate and 10.3 g of disodium EDTA dihydrate in 800 mL of water, add 2 volumes of 6 N acetic acid to 1 volume of water, and adjust the pH to 5.0 with 50% potassium hydroxide solution, and dilute with water to volume, and mix. Store this stock solution in a plastic bottle. On the day of use, transfer 5.0 mL of the stock solution into a 1000-mL volumetric flask; dilute with water to volume, and mix.

Calibration curve: Transfer 1.0, 2.0, 3.0, 5.0, 10.0, and 15.0 mL of the Sodium fluoride solution into separate 250-mL plastic beakers; add 50 mL of water, 5 mL of 1 N hydrochloric acid, 10 mL of 1 M sodium citrate, and 10 mL of 0.2 M disodium EDTA to each beaker; and mix. Transfer each solution into separate 100-mL volumetric flasks, dilute with water to volume, and mix. Transfer a 50-mL portion of each solution into separate 125-mL plastic beakers, and measure the potential of each solution with a suitable ion-selective electrode apparatus (such as the Orion Model No. 94-09, with solid-state membrane), using a suitable reference electrode (such as the Orion Model No. 90-01, with single junction). Plot the Calibration curve on two-cycle semilogarithmic paper (such as K & E No. 465130) or with the use of a suitable graphing calculator or spreadsheet program, with µg of fluoride (F) per 100 mL of solution on the logarithmic scale.

Procedure: Transfer 1.00 g of sample into a 150-mL glass beaker, add 10 mL of water, and, while stirring continuously, slowly add 20 mL of 1 N hydrochloric acid to dissolve the sample. Boil rapidly for 1 min, then transfer into a 250-mL plastic beaker, and cool rapidly in ice water. Add 15 mL of 1 M sodium citrate and 10 mL of 0.2 M disodium EDTA, and mix. Adjust the pH to 5.5 ± 0.1 with 1 N hydrochloric acid or 1 N sodium hydroxide, if necessary; transfer into a 100-mL volumetric flask; dilute with water to volume; and mix. Transfer a 50-mL portion of this solution into a 125-mL plastic beaker, and measure the potential of the solution with the apparatus described under Calibration curve. Determine the fluoride content, in µg, of the sample from the Calibration curve.

Determine the percentage of fluoride in the sample taken:

\[
\text{Result} = \left( \frac{800}{W} \right) \left( \frac{1.01 \times 10^{(E_2 - E_1)/S}}{1} \right)
\]

800 = factor that corrects for the sample dilutions
W = original weight of the sample (g)
1.01 = correction factor that is the relationship between the volume of standard used for standard addition and the volume of the sample dilution on which the standard addition is performed (V_s/V)
E2 = final equilibrated reading (mV)
E1 = initial equilibrated reading (mV)
S = electrode slope

• METHOD III (ION-SELECTIVE ELECTRODE METHOD B)
Sodium fluoride solution (5 µg F/mL): Transfer 2.210 g of sodium fluoride, previously dried at 200° for 4 h and accurately weighed, into a 400-mL plastic beaker, add 200 mL of water, and stir until dissolved. Quantitatively transfer this solution into a 1000-mL volumetric flask with the aid of water, dilute with water to volume, and mix. Store this stock solution in a plastic bottle. On the day of use, transfer 5.0 mL of the stock solution into a 1000-mL volumetric flask, and dissolve in and dilute with water to volume. The resulting solution contains 1000 µg of fluoride per mL.

Procedure: Transfer 1.00 g of sample into a 150-mL glass beaker, add 20 mL of water, and, while stirring continuously, slowly add 20 mL of 1 N hydrochloric acid to dissolve the sample. Boil rapidly for 1 min, then transfer into a 250-mL plastic beaker, and cool rapidly in ice water. Add 15 mL of 1 M sodium citrate and 10 mL of 0.2 M disodium EDTA, and mix. Adjust the pH to 5.5 ± 0.1 with 1 N hydrochloric acid or 1 N sodium hydroxide, if necessary; transfer into a 100-mL volumetric flask; dilute with water to volume; and mix. Transfer a 50-mL portion of this solution into a 125-mL plastic beaker, and measure the potential of the solution with the apparatus described under Calibration curve. Determine the fluoride content, in µg, of the sample from the Calibration curve.

Determine the percentage of fluoride in the sample taken:

\[
\text{Result} = \left( \frac{C}{W_s} \right) \times 0.000001 \times 100\%
\]

C = content of fluoride in the sample, determined from the Calibration curve (µg)
W_s = sample weight (g)
0.000001 = conversion factor (µg to grams)

• METHOD IV (ION-SELECTIVE ELECTRODE METHOD C)

Buffer solution A: Add 2 volumes of 6 N acetic acid to 1 volume of water, and adjust the pH to 5.0 with 50% potassium hydroxide solution.

Buffer solution B: Dissolve 150 g of sodium citrate dihydrate and 10.3 g of disodium EDTA dihydrate in 800 mL of water, adjust the pH to 8.0 with 50% sodium hydroxide solution, and dilute with water to 1000 mL.

Fluoride standard solutions
1000 mg/kg Fluoride standard: Transfer 2.2108 g of sodium fluoride, previously dried at 200° for 4 h, into a 1000-mL volumetric flask, and dissolve in and dilute with water to volume. The resulting solution contains 1000 µg of fluoride per mL.

50 mg/kg Fluoride standard: Pipet 50 mL of the 1000 mg/kg Fluoride standard into a 1000-mL volumetric flask. Dilute with water to volume.

10 mg/kg Fluoride standard: Pipet 100 mL of the 50 mg/kg Fluoride standard into a 500-mL volumetric flask. Dilute with water to volume.

Fluoride limit solutions (for a 1-g sample)
50 mg/kg Fluoride limit solution (1 mg/kg Fluoride standard): Pipet 50 mL of the 10 mg/kg Fluoride standard into a 500-mL volumetric flask, and dilute with water to volume.
Lime suspension:

Pipet 50 mL of the
Fluoride limit solution:
Buffer solution:
Fluoride standard:
Sample preparation:

Electrode calibration:
Pipet 50 mL of the appropriate Buffer solution into a plastic beaker. Place the fluoride ion and reference electrodes (or a combination fluoride electrode) into the plastic beaker and stir. At 5-min intervals, add 100 µL and 1000 µL of the 1000 mg/kg Fluoride standard and read the potential, in mV, after each addition. The difference between the two readings is the slope of the fluoride electrode and should typically be in the range of 63–70 mV at 25° for Buffer solution A and in the range of 54–60 mV at 25° for Buffer solution B. If the difference in potential is not within this range, check, and, if necessary, replace the electrode, instrument, or solutions.

Alternatively, the electrode calibration should be performed according to the manufacturer’s instructions and should comply with the manufacturer’s calibration range at 25°. If the difference in potential is not within this range, evaluate the system and equipment as necessary.

Procedure: Transfer the entire sample into a plastic beaker. Place the fluoride ion and reference electrodes (or a combination fluoride electrode) into the plastic beaker and stir. After 5 min, add 100 µL and 1000 µL of the 1000 mg/kg Fluoride standard and read the potential, in mV, after each addition. The difference between the two readings is the slope of the fluoride electrode and should typically be in the range of 63–70 mV at 25° for Buffer solution A and in the range of 54–60 mV at 25° for Buffer solution B. If the difference in potential is not within this range, check, and, if necessary, replace the electrode, instrument, or solutions.

Alternatively, the electrode calibration should be performed according to the manufacturer’s instructions and should comply with the manufacturer’s calibration range at 25°. If the difference in potential is not within this range, evaluate the system and equipment as necessary.

Procedure: Transfer the entire sample into a plastic beaker. Place the fluoride ion and reference electrodes (or a combination fluoride electrode) into the plastic beaker and stir. After 5 min, add 100 µL and 1000 µL of the 1000 mg/kg Fluoride standard and read the potential, in mV, after each addition. The difference between the two readings is the slope of the fluoride electrode and should typically be in the range of 63–70 mV at 25° for Buffer solution A and in the range of 54–60 mV at 25° for Buffer solution B. If the difference in potential is not within this range, check, and, if necessary, replace the electrode, instrument, or solutions.

Alternatively, the electrode calibration should be performed according to the manufacturer’s instructions and should comply with the manufacturer’s calibration range at 25°. If the difference in potential is not within this range, evaluate the system and equipment as necessary.
to the manufacturer’s instructions and should comply with the manufacturer’s calibration range at 25°. If the difference in potential is not within this range, evaluate the system and equipment as necessary.

**Analysis:** Transfer the sample into a 100-mL volumetric flask, and dissolve it in a minimal amount of water. Add 50.0 mL of the Buffer solution, dilute with water to volume, and mix. Transfer the entire solution to a plastic beaker. Place the fluoride ion and reference electrode (or fluoride combination electrode) into the beaker, allow the solution to equilibrate for 5 min with stirring, and read the potential in mV (E₁). Pipet 0.25 mL of the Fluoride standard solution into the beaker, allow the electrode to come to equilibrium, and record the final potential in mV (E₂). Measure a blank fluoride level by repeating this procedure without sample and using 0.1 mL of the Fluoride standard solution.

Calculate the change in mV (ΔE) for the blank and the sample taken:

$$\Delta E = E_1 - E_2$$

\[E_1 = \text{reading before addition of the Fluoride standard solution (mV)}\]
\[E_2 = \text{reading after addition of the Fluoride standard solution (mV)}\]

Calculate the diluted fluoride level, in µg/mL, in the blank (C₉) and in the sample taken (C₈):

$$C_9 = 0.25/(1.0025 \times 10^{\Delta E/5} - 1)$$

$$C_8 = 0.1/(1.001 \times 10^{\Delta E/5} - 1)$$

0.25, 1.0025, 0.1, and 1.001 = formula constants

\[\Delta E = \text{change in mV calculated above}\]

\[S = \text{absolute value of the electrode as determined in Electrode Calibration}\]

Calculate the fluoride content, in mg/kg, in the sample taken:

$$\text{Result} = 100 \times (C_S - C_B)/W$$

C₉ = diluted fluoride level in the Sample calculated above (µg/mL)

C₈ = diluted fluoride level in the blank calculated above (µg/mL)

W = weight of the Sample (g)

**LEAD LIMIT TEST**

[Note—Unless otherwise specified in the monograph, use the Dithizone Method to determine lead levels.]

• **DITHIZONE METHOD**

**Special reagents:** Select reagents having as low a lead content as practicable, and store all solutions in containers of borosilicate glass. Rinse all glassware thoroughly with warm, 1:2 nitric acid followed by water.

**Ammonia–cyanide solution:** Dissolve 2 g of potassium cyanide in 15 mL of ammonium hydroxide, and dilute with water to 100 mL.

**Ammonium citrate solution:** Dissolve 40 g of citric acid in 90 mL of water, add 2 or 3 drops of phenol red TS, then cautiously add ammonium hydroxide until the solution acquires a red color. Extract it with 20-mL portions of Dithizone extraction solution until the dithizone solution retains its green color or remains unchanged.

**Diluted standard lead solution (1 µg Pb in 1 mL)**

Lead nitrate stock solution: Dissolve 159.8 mg of ACS Reagent-Grade Lead Nitrate [Pb(NO₃)₂] in 100 mL of water containing 1 mL of nitric acid, dilute with water to 1000.0 mL, and mix. Prepare and store this solution in glass containers that are free from lead salts.

Standard lead solution: On the day of use, dilute 10.0 mL of Lead nitrate stock solution with water to 100.0 mL. Each mL of Standard lead solution contains the equivalent of 10 µg of lead (Pb) ion.

Diluted standard lead solution: Immediately before use, transfer 10.0 mL of Standard lead solution into a 100-mL volumetric flask, dilute with 1:100 nitric acid to volume, and mix.

Dithizone extraction solution: Dissolve 20 g of dithizone in 1000 mL of chloroform, add 5 mL of alcohol, and mix. Store in a refrigerator. Before use, shake a suitable volume of the solution with about half its volume of 1:100 nitric acid, discarding the nitric acid.

Hydroxylamine hydrochloride solution: Dissolve 20 g of hydroxylamine hydrochloride in sufficient water to make about 65 mL, transfer the solution into a separator, add a few drops of thymol blue TS, then add ammonium hydroxide until the solution assumes a yellow color. Add 10 mL of a 1:25 solution of sodium diethylthiocarbamate, mix, and allow to stand for 5 min. Extract the solution with successive 10- to 15-mL portions of chloroform until a 5-mL test portion of the chloroform extract does not assume a yellow color when shaken with cupric sulfate TS. Add 2.7 N hydrochloric acid until the extracted solution is pink, adding 1 or 2 drops more of thymol blue TS if necessary, then dilute with water to 100 mL, and mix.

Potassium cyanide solution: Dissolve 50 g of potassium cyanide in sufficient water to make 100 mL. Remove the lead from the solution by extraction with successive portions of Dithizone extraction solution as described under Ammonium citrate solution, then extract any dithizone remaining in the cyanide solution by shaking with chloroform. Finally, dilute the cyanide solution with sufficient water so that each 100 mL contains 10 g of potassium cyanide.
Standard dithizone solution: Dissolve 10 mg of dithizone in 1000 mL of chloroform, keeping the solution in a glass-stoppered, lead-free bottle suitably wrapped to protect it from light and stored in a refrigerator.

Sample solution: Use the solution obtained by treating the sample as directed in the individual monograph as the Sample solution in the Procedure. Sample solutions of organic compounds are prepared, unless otherwise directed, according to the following general method: [CAUTION—Some substances may react unexpectedly with explosive violence when digested with hydrogen peroxide. Use appropriate safety precautions at all times.]

Transfer 1.0 g of sample into a suitable flask, add 5 mL of sulfuric acid and a few glass beads, and digest at a temperature not exceeding 120° until charring begins, using preferably a hot plate in a fume hood. (Additional sulfuric acid may be necessary to completely wet some samples, but the total volume added should not exceed about 10 mL.) After the sample has initially been decomposed by the acid, add with caution, dropwise, hydrogen peroxide (30%), allowing the reaction to subside and reheating between drops. The first few drops must be added very slowly with sufficient mixing to prevent a rapid reaction, and heating should be discontinued if foaming becomes excessive. Swirl the solution in the flask to prevent unaeracted substance from caking on the walls or bottom of the flask during the digestion.

[NOTE—Add small quantities of the peroxide when the solution begins to darken.]

Continue the digestion until the organic matter is destroyed, gradually raising the temperature of the hot plate to 250°–300° until fumes of sulfur trioxide are copiously evolved and the solution becomes colorless or retains only a light straw color. Cool, cautiously add 10 mL of water, again evaporate to strong fuming, and cool. Quantitatively transfer the solution into a separator with the aid of small quantities of water.

Procedure: Transfer the Sample solution, prepared as directed in the individual monograph, into a separator, and unless otherwise directed, add 6 mL of Ammonium citrate solution and 2 mL of Hydroxylamine hydrochloride solution. (Use 10 mL of the citrate solution when determining lead in iron salts.) Add 2 drops of phenol red TS to the separator, and make the solution just alkaline (red in color) by the addition of ammonium hydroxide. Cool the solution, if necessary, under a stream of tap water, then add 2 mL of Potassium Cyanide Solution. Immediately extract the solution with 5-mL portions of Dithizone extraction solution, draining each extract into another separator, until the dithizone solution retains its green color. Shake the combined dithizone solutions for 30 s with 20 mL of 1:100 nitric acid, discard the chloroform layer, add 5.0 mL of Standard Dithizone Solution and 4 mL of Ammonia–cyanide solution to the acid solution, and shake for 30 s. The purple hue in the chloroform solution of the sample caused by any lead dithizionate present does not exceed that in a control, containing the volume of Diluted Standard lead solution equivalent to the amount of lead specified in the monograph, when treated in the same manner as the sample.

**Flame Atomic Absorption Spectrophotometric Method**

Select reagents having as low a lead content as practicable, and store all solutions in high-density polyethylene containers. Rinse all plastic and glassware thoroughly with warm 1:2 nitric acid followed by water.

Lead nitrate stock solution (100 µg/mL): Dissolve 159.8 mg of reagent-grade lead nitrate [Pb(NO$_3$)$_2$] in 100 mL of water containing 1 mL of nitric acid in a 1000-mL volumetric flask, and dilute with water to volume.

Standard lead solution (10 µg/mL): On the day of use, transfer 10 mL of Lead nitrate stock solution into a 100-mL volumetric flask, and dilute with water to volume.

Diluted standard lead solutions: On the day of use, prepare a set of Standard lead solutions that corresponds to the lead limit specified in the monograph:

- 1 mg/kg Lead limit (0.5 µg/mL, 1.0 µg/mL, and 1.5 µg/mL standards): On the day of use, transfer 5.0 mL, 10.0 mL, and 15.0 mL of Standard lead solution into three separate 100-mL volumetric flasks, add 10 mL of 3 N hydrochloric acid to each, and dilute with water to volume.
- 5 mg/kg Lead limit (1.0 µg/mL, 5.0 µg/mL, and 10.0 µg/mL standards): On the day of use, transfer 10.0 mL and 50.0 mL of Standard lead solution into two separate 100-mL volumetric flasks, add 10 mL of 3 N hydrochloric acid to each, and dilute with water to volume. The final standard, 10.0 µg/mL, is taken directly from the Standard lead solution.
- 10 mg/kg Lead limit (5.0 µg/mL, 10.0 µg/mL, and 15.0 µg/mL standards): On the day of use, transfer 5.0 mL, 10.0 mL, and 15.0 mL of Lead nitrate stock solution into three separate 100-mL volumetric flasks, add 10 mL of 3 N hydrochloric acid to each, and dilute with water to volume.
- 25% Sulfuric acid solution (by volume): Carefully add 100 mL of sulfuric acid to 300 mL of water with constant stirring while cooling in an ice bath.

Sample preparation: Transfer the sample weight as specified in the monograph, weighed to the nearest 0.1 mg, into an evaporating dish. Add a sufficient amount of 25% Sulfuric acid solution, and distribute the sulfuric acid uniformly through the sample. Within a hood, place the dish on a steam bath to evaporate most of the water. Place the dish on a burner, and slowly pre-ash the sample by expelling most of the sulfuric acid. Place the dish in a muffle furnace that has been set at 525°, and ash the sample until the residue appears free from carbon. Prepare a Sample Blank by ashing 5 mL of 25% sulfuric acid. Cool, and cautiously wash down the inside of each evaporation dish with water.

Add 5 mL of 1 N hydrochloric acid. Place the dish on a steam bath, and evaporate to dryness. Add 1.0 mL of 3 N hydrochloric acid and approximately 5 mL of water, and heat briefly on a steam bath to dissolve any residue.

Transfer each solution quantitatively to a 10-mL volumetric flask, dilute to volume, and mix.

Procedure: Concomitantly determine the absorbances of the Sample Blank, the Diluted standard lead solutions, and the Sample preparation at the lead emission line of 283.3 nm, using a slit-width of 0.7 nm. Use a suitable atomic absorption spectrophotometer equipped with a lead electrodeless discharge lamp (EDL), an air–acetylene flame, and a 4-in burner head, water as the blank.

Calculations: Determine the corrected absorbance values by subtracting the Sample Blank absorbance from each of the Diluted standard lead solutions and from the Sample preparation absorbances. Prepare a Standard curve by plotting the corrected Diluted standard lead solutions absorbance values versus their corresponding concentrations expressed as µg/
ml. Determine the lead concentration in the Sample preparation by reference to the calibration curve. Calculate the quantity of lead, in mg/kg, in the sample taken:

\[
\text{Result} = 10C/W_s
\]

\[
C = \text{concentration of lead from the standard curve (µg/mL)}
\]

\[
W_s = \text{weight of the sample taken (g)}
\]

**ATOMIC ABSORPTION SPECTROPHOTOMETRIC GRAPHITE FURNACE METHOD**

The following methods are primarily intended for the analysis of applicable substances containing less than 1 mg/kg of lead.

**Method I**

This method is intended for the quantitation of lead in substances that are soluble in water, such as sugars and sugar syrups, at levels as low as 0.03 mg/kg. The method detection limit is approximately 5 ng/kg.

**Apparatus:** Use a suitable graphite furnace atomic absorption spectrophotometer set at 283.3 nm and equipped with an autosampler, pyrolytically coated graphite tubes, solid pyrolytic graphite platforms, and an adequate means of background correction. Zeeman effect or Smith-Hieftje background correction is preferred, but deuterium arc background correction should be acceptable. (This method was developed on a Perkin-Elmer Model ZS100, 0.7-nm slit, HGA-600 furnace, AS-60 autosampler with Zeeman background correction.) If the instrument does not have a well-defined calibration function, a separate calculator or computer is required for linear least squares, nonlinear, or quadratic calibrations. Set up the instrument according to the manufacturer’s specifications with consideration of current good GFAAS practices—addressing such factors as line voltage, cooling water temperature, graphite part specifications, and furnace temperature. If an optical pyrometer or thermocouple is not available to check the furnace controller temperature calibration, dim the room lights, and observe the furnace emission through the sample introduction port while increasing the furnace temperature. A characteristic cherry red glow should begin to appear at 800°. If it glows at a lower temperature, then the furnace is hotter, and temperatures must be adjusted downward accordingly.

Use acid-cleaned [in a mixture of 5% sub-boiling, distilled nitric acid and 5% sub-boiling, distilled hydrochloric acid made up in deionized, distilled water (18 megohm), and thoroughly rinsed with deionized, distilled water (18 megohm)] autosampler cups (PE B008-7600 Teflon, or equivalent) to avoid contamination. Use micropipets with disposable tips free of lead contamination for dilution. Ensure accuracy and precision of micropipets and tips by dispensing and weighing 5–10 replicate portions of water onto a microbalance. Use acid-cleaned volumetric glassware to prepare standards and dilute samples to a final volume. For digestion, use acid-cleaned, high-density polyethylene tubes, polypropylene tubes, Teflon tubes, or quartz tubes. Store final diluted samples in plastic tubes.

**Air ashing:** The furnace controller must be able to handle 2 gas flows to facilitate air ashing. Oxygen ashing is used to avoid build up of residue during the char step. Argon is used as the purge gas for the furnace for all steps but the char. Breathing quality air can be used as the alternate gas for the air ashing.

**Standard solutions:** Prepare all lead solutions in 5% sub-boiling distilled nitric acid. Use a single-element 1000- or 10,000-µg/mL lead stock to prepare (weekly) an intermediate 10-µg/mL standard in 5% nitric acid. Prepare (daily) a Lead standard solution (1 µg/mL) by diluting the intermediate 10-µg/mL stock solution 1:10. Prepare Working Calibration Standards of 100.0 ng/mL, 50.0 ng/mL, 25.0 ng/mL, and 10.0 ng/mL from this, using appropriate dilutions. Store standards in acid-cleaned polyethylene test tubes or bottles. If the GFAAS autosampler is used to automatically dilute standards, ensure calibration accuracy by pipetting volumes of 3 µL or greater.

**Modifier stock solution:** Weigh 20 g of ultrapure magnesium nitrate hexahydrate, and dilute to 100 mL. Just before use, prepare a Modifier working solution by diluting stock solution 1:10. A volume of 5 µL will provide 0.06 mg of magnesium nitrate.

**Sample digestion**

**[Caution—Perform the procedure in a fume hood, and wear safety glasses.]**

Obtain a representative subsample to be analyzed. For liquid samples such as sugar syrups, ultrasonicate and/or vortex mix before weighing. For solid samples such as crystalline sucrose, make a sugar solution using equal weights of sample (5-g minimum) and deionized, distilled (18 megohm) water. Mix samples until completely dissolved. Transfer approximately 1.5 g (record to nearest mg) of sample (or 3.0 g of sugar solution), accurately weighed, into a digestion tube. Run a Sample preparation blank of 1.5 g of deionized, distilled (18 megohm) water through the entire procedure with each batch of samples. Add 0.75 mL of sub-boiling, distilled nitric acid. Heat plastic tubes in a water bath, quartz tubes in a water bath or heating block, warming slowly to 90°–95° to avoid spattering. Monitor the temperature by using a “dummy” sample. Heat until all brown vapors have dissipated and any rust-colored tint is gone (20–30 min). Cool. Add 0.5 mL of 50% hydrogen peroxide dropwise, heat at 90°–95° for 5 min, and cool. Add a second 0.5-mL portion of 50% hydrogen peroxide, dropwise, and heat at 90°–100° for 5–10 min or until clear. Cool, and dilute with water to a final volume of 10 mL.

**Procedure:** The furnace program is as follows: (1) Dry at 200°, using a 20-s ramp and a 30-s hold and a 300-mL/min argon flow; (2) char the sample at 750°, using a 40-s ramp and a 40-s hold and a 300-mL/min air flow [Note—Air ashing is a critical step to avoid build up of residue during the char step.]; (3) cool down, and purge the air from the furnace for 60 s, using a 20° set temperature and a 300-mL/min argon flow; (4) atomize at 1800°, using a 0-s ramp and a 10-s hold with the argon flow stopped; (5) clean out at 2600°, with a 1-s ramp and a 7-s hold; (6) cool down the furnace (if necessary) at 20°, with a 1-s ramp and a 5-s hold with a 300-mL/min argon flow.

Program the autosampler to add 5 µL of Modifier working solution separately into 20 µL each of blanks, calibration standards, and sample solutions while introducing the solutions into the graphite furnace. Inject each respective solution in triplicate, and average the results. Use peak area measurements for all quantitation. After ensuring that
the furnace is clean by running a 5% nitric acid blank, check the instrument sensitivity according to manufacturer’s specifications by running the 25-ng/mL calibration standard. Calculate the characteristic mass \( m_c \) (mass of Pb pg necessary to produce an integrated absorbance of 0.0044 abs-sec) as follows:

\[
m_c = (0.0044 \text{ abs-sec})(25 \text{ pg/µL})(20 \text{ µL})/(\text{measured 25 pg/µL abs-sec})
\]

Record and track the integrated absorbance and \( m_c \) for reference and quality assurance.

**Standard curve:** Inject each calibration standard in triplicate and determine the instrument linearity according to manufacturer’s instructions. Use the calibration algorithms provided in the instrument software. Recheck calibration periodically (≤15 samples) by running a 25- or 50-ng/mL calibration standard interspersed with samples. If recheck differs from calibration by >10%, recalibrate the instrument. The instrumental detection limit (DL) and quantitation limit (QL), in picograms, may be based on 7–10 replicates of the Sample preparation blank and calculated as follows:

\[
\text{DL} = (3)(\text{s.d. blank abs-sec})(10 \text{ pg/µL})(20 \text{ µL})/(\text{abs-sec 10 ng/mL std})
\]

\[
\text{QL} = (10)(\text{s.d. blank abs-sec})(10 \text{ pg/µL})(20 \text{ µL})/(\text{abs-sec 10 ng/mL std})
\]

During method development, detection limits were typically 10–14 pg, corresponding to 0.5–0.7 ng/mL for 20 µL. This corresponds to a method detection limit of 3.3–4.7 ng/g of sugar.

**Sample analyses:** Inject each sample digest in triplicate, and record the integrated absorbance. If instrument response exceeds that of the calibration curve, dilute with 5% nitric acid to bring the sample response into working range, and note the dilution factor (DF). Sample solutions having a final concentration beyond the linearity range should be diluted 1:10 to facilitate analysis in the linear range for systems not equipped with nonlinear calibration. All sample solutions should be blank corrected using the Sample preparation blank. This can typically be done automatically by the software after identifying and running a representative Sample preparation blank. Use the calibration algorithm provided in the instrument software to calculate a blank-corrected, digest lead concentration (in ng/mL).

**Calculation of lead content:** Calculate the lead level in the original sample as follows:

\[
Pb (\text{ng/g}) = (\text{blank-corrected Pb ng/mL})(\text{DF})(\text{sample vol (10 mL)})/(\text{sample wt (approx. 1.5 g)})^2
\]

**Quality assurance:** To ensure analytical accuracy, an appropriate trace elements in water reference standard with a certified lead content or a similar material should be analyzed before the unknown samples are.\(^2\) If the concentration determined is not within 10% of the mean reference value, the reason for inaccuracy should be evaluated, and unknown samples should not be analyzed until acceptable accuracy is achieved. Also prepare an in-house control solution made from uncontaminated table sugar or reagent-grade sucrose (or other appropriate substance with a Pb content <5 ng/g as received) mixed with an equal volume of water. Spike this solution with Pb to produce a concentration of 100 ng/g. Analyze with each batch of samples. Recoveries should be 100 ± 20%, and the precision for complete replicate digestions should be <5% RSD. Periodically, a sample digest should be checked using the method of standard additions to ensure that there are no multiplicative or chemical interferences. Spiking samples and checking recoveries is always a good practice.

**Method II**

This method is primarily intended for the determination of lead at levels of less than 1 mg/kg in substances immiscible with water, such as edible oils.

**Apparatus:** Use a suitable atomic absorption spectrophotometer (Perkin-Elmer Model 3100 or equivalent) fitted with a graphite furnace (Perkin-Elmer HGA 600 or equivalent). Use a lead hollow-cathode lamp (Perkin-Elmer or equivalent) with argon as the carrier gas. Follow the manufacturers’ directions for setting the appropriate instrument parameters for lead determination.

**Hydrogen peroxide–nitric acid solution:** Dissolve equal volumes of 10% hydrogen peroxide and 10% nitric acid.

**Lead nitrate stock solution:** Dissolve 159.8 mg of ACS Reagent-Grade Lead Nitrate (alternatively, use NIST Standard Reference Material, containing 10 mg of lead per kg) in 100 mL of Hydrogen peroxide–nitric acid solution. Dilute with Hydrogen peroxide–nitric acid solution to 1000.0 mL, and mix. Prepare and store this solution in glass containers that are free from lead salts. Each mL of this solution contains the equivalent of 100 µg of lead (Pb) ion.

**Standard lead solution:** On the day of use, dilute 10.0 mL of Lead nitrate stock solution with Hydrogen peroxide–nitric acid solution to 100.0 mL, and mix. Each mL of Standard lead solution contains the equivalent of 1 µg of lead (Pb) ion.

**Butanol–nitric acid solution:** Slowly add 50 mL of nitric acid to approximately 500 mL of butanol contained in a 1000- mL volumetric flask. Dilute with butanol to volume, and mix.

**Standard solutions:** Prepare a series of Lead standard solutions serially diluted from the Standard lead solution in Butanol–nitric acid solution. Pipet into separate 100-mL volumetric flasks 0.2 mL, 0.5 mL, 1 mL, and 2 mL, respectively, of

\[^2\] If a Sample solution was prepared initially to ensure sample homogeneity, this is the weight of the original sugar digested (not the weight of the solution).
\[^3\] NIST SRM 1643 - Trace Elements in Water (National Institute of Standards and Technology, Gaithersburg, MD, USA), or equivalent.
Standard lead solution, dilute with Butanol–nitric acid solution to volume, and mix. The Standard solutions contain, respectively, 0.02 μg, 0.05 μg, 0.1 μg, and 0.2 μg of lead per mL. (For lead limits greater than 1 mg/kg, prepare a series of Standard solutions in a range encompassing the expected lead concentration in the sample.)

Sample solution

[CAUTION—Perform this procedure in a fume hood, and wear safety glasses.]

Transfer 1 g of sample, accurately weighed, into a large test tube. Add 1 mL of nitric acid. Place the test tube in a rack in a boiling water bath. As soon as the rusty tint is gone, add 1 mL of 30% hydrogen peroxide dropwise to avoid a vigorous reaction, and wait for bubbles to form. Stir with an acid-washed plastic spatula if necessary. Remove the test tube from the water bath, and let it cool. Transfer the solution into a 10-mL volumetric flask, and dilute with Butanol–nitric acid solution to volume, and mix. Use this solution for analysis.

Procedure:

Tungsten solution: Transfer 0.1 g of tungstic acid (H₂WO₄) and 5 g of sodium hydroxide pellets into a 50-mL plastic bottle. Add 5.0 mL of high-purity water, and mix. Heat the mixture in a hot water bath until a complete solution is achieved. Cool, and store at room temperature.

Procedure: Place the graphite tube in the furnace. Inject a 20-μL aliquot of the Tungsten solution into the graphite tube, using a 300-μL/min argon flow and the following sequence of conditions: dry at 110° for 20 s, char at 700°–900° for 20 s, and with the argon flow stopped, atomize at 2700° for 10 s; repeat this procedure once more using a second 20-μL aliquot of the Tungsten solution. Clean the quartz windows.

Standard curve: [Note—The sample injection technique is the most crucial step in controlling the precision of the analysis. The volume of the sample must remain constant. Rinse the 1-μL pipet tip (Eppendorf or equivalent) three times with either the Standard solutions or Sample solution before injection. Use a fresh pipet tip for each injection, and start the atomization process immediately after injecting the sample. Between injections, flush the graphite tube of any residual lead by purging at a high temperature as recommended by the manufacturer.]

With the hollow-cathode lamp properly aligned for maximum absorbance and the wavelength set at 283.3 nm, atomize 20-μL aliquots of the four Standard solutions, using a 300-μL/min argon flow and the following sequence of conditions: dry at 110° for 30 s, with a 20-s ramp period and a 10-s hold time; then char at 700° for 42 s, with a 20-s ramp period and a 22-s hold time; and then, with the argon flow stopped, atomize at 2300° for 7 s. Plot a Standard curve using the concentration, in μg/mL, of each Standard solution versus its maximum absorbance value compensated for background correction as directed for the particular instrument, and draw the best straight line. Atomize 20 μL of the Sample solution under identical conditions, and measure its corrected maximum absorbance. From the Standard curve, determine the concentration, C, in μg/mL, of the Sample solution. Calculate the quantity, in mg/kg, of lead in the sample:

\[
\text{Result} = \frac{10C}{W}
\]

in which W is the weight, in grams, of the sample taken.

• APDC EXTRACTION METHOD

Select reagents having as low a lead content as practicable, and store all solutions in high-density polyethylene containers. Rinse all plastic and glassware thoroughly with warm, 1:2 nitric acid followed by water.

2% APDC solution: Dissolve 2.0 g of ammonium pyrrolidinedithiocarbamate (APDC) in 100 mL of water. Filter any slight residue of insoluble APDC from the solution before use.

Lead nitrate stock solution (100 μg/mL): Dissolve 159.8 mg of reagent-grade lead nitrate [Pb(NO₃)₂] in 100 mL of water containing 1 mL of nitric acid in a 1000-mL volumetric flask, and dilute with water to volume.

Standard lead solutions

2 mg/kg Lead standard: On the day of use, transfer 2.0 mL of Lead nitrate stock solution into a 100-mL volumetric flask, and dilute with water to volume. The resulting solution contains 2 μg of lead per mL.

3 mg/kg Lead standard: On the day of use, transfer 3.0 mL of Lead nitrate stock solution into a 100-mL volumetric flask, and dilute with water to volume. The resulting solution contains 3 μg of lead per mL.

4 mg/kg Lead standard: On the day of use, transfer 4.0 mL of Lead nitrate stock solution into a 100-mL volumetric flask, and dilute with water to volume. The resulting solution contains 4 μg of lead per mL.

10 mg/kg Lead standard: On the day of use, transfer 10.0 mL of Lead nitrate stock solution into a 100-mL volumetric flask, and dilute with water to volume. The resulting solution contains 10 μg of lead per mL.

Sample preparation: Transfer a 10.0-g sample to a clean 150-mL beaker. Add 30 mL of water and the minimum amount of hydrochloric acid needed to dissolve the sample, plus an additional 1 mL of hydrochloric acid to ensure the dissolution of any lead present. Heat to boiling, and boil for several minutes. Allow to cool, and dilute with deionized water to about 100 mL. Adjust the pH of the resulting solution to 1.0–1.5 with 25% NaOH. Quantitatively transfer the pH-adjusted solution to a clean 250-mL separatory funnel, and dilute with water to about 200 mL. Add 2 mL of 2% APDC Solution, and mix. Extract with two 20-mL portions of chloroform, collecting the extracts in a clean 50-mL beaker. Evaporate to dryness on a steam bath. Add 3 mL of nitric acid to the residue, and heat near dryness. Then add 0.5 mL of nitric acid and 10 mL of deionized water to the beaker, and heat until the volume is reduced to about 3–5 mL. Transfer the digested extract to a clean 10-mL volumetric flask, and dilute with water to volume.

Reagent blank: Prepare as Sample preparation, except do not add the 10 g of sample, and replace 10 g of sample with water.

Procedure: Zero the instrument with water, and concomitantly determine the absorbances of the appropriate Standard lead solution, the Sample preparation, and the Reagent blank at the lead emission line of 283.3 nm, using a slit-width of 0.7 nm. Use a suitable atomic absorption spectrophotometer equipped with a lead electrodeless discharge lamp (EDL), or equivalent; an air–acetylene flame; and a 4-in burner head. Correct the absorbance of the Sample preparation with
the absorbance obtained from the Reagent blank. The absorbance of the corrected Sample preparation is not greater than that of the Standard lead solution.

**MANGANESE LIMIT TEST**

**Manganese detection instrument:** Use any suitable atomic absorption spectrophotometer equipped with a fast-response recorder or other readout device and capable of measuring the radiation absorbed by manganese atoms at the manganese resonance line of 279.5 nm.

**Standard preparations:** Transfer 1000 mg, accurately weighed, of manganese metal powder into a 1000-mL volumetric flask, dissolve by warming in a mixture of 10 mL of water and 10 mL of 0.5 N hydrochloric acid, cool, dilute with water to volume, and mix. Pipet 5.0 mL of this solution into a 50-mL volumetric flask, dilute with water to volume, and mix. Finally, pipet 5.0 mL, 10.0 mL, 15.0 mL, and 25.0 mL of this solution into separate 1000-mL volumetric flasks, dilute each flask with water to volume, and mix. The final solutions contain 0.5 mg/kg, 1.0 mg/kg, 1.5 mg/kg, and 2.5 mg/kg of Mn, respectively.

**Sample preparation:** Transfer 10.000 g of the sample into a 200-mL Kohlrausch volumetric flask, previously rinsed with 0.5 N hydrochloric acid, add 140 mL of 0.5 N hydrochloric acid, and shake vigorously for 15 min, preferably with a mechanical shaker. Dilute with 0.5 N hydrochloric acid to volume, and shake. Centrifuge approximately 100 mL of the sample mixture in a heavy-walled centrifuge tube at 2000 rpm for 5 min, and use the clear supernatant liquid in the following Procedure.

**Procedure:** Aspirate 0.5 N hydrochloric acid through the air–acetylene burner for 5 min, and obtain a baseline reading at 279.5 nm, following the manufacturer’s instructions for operating the atomic absorption spectrophotometer being used for the analysis. Aspirate a portion of each Standard preparation in the same manner, note the readings, then aspirate a portion of the Sample preparation, and note the reading. Prepare a Standard curve by plotting the mg/kg of Mn in each Standard preparation against the respective readings. From the graph determine the mg/kg of Mn in the Sample preparation, and multiply this value by 20 to obtain the mg/kg of Mn in the original sample taken for analysis.

**MERCURY LIMIT TEST**

**Method I**

**Mercury detection instrument:** Use any suitable atomic absorption spectrophotometer equipped with a fast-response recorder and capable of measuring the radiation absorbed by mercury vapors at the mercury resonance line of 253.6 nm. A simple mercury vapor meter or detector equipped with a variable span recorder also is satisfactory.

[NOTE—Wash all glassware associated with the test with nitric acid, and rinse thoroughly with water before use.]

**Aeration apparatus:** The apparatus, shown in Figure 6, consists of a flowmeter (a), capable of measuring flow rates from 500 to 1000 mL/min, connected via a three-way stopcock (b), with a Teflon plug, to 125-mL gas washing bottles (c and d), followed by a drying tube (e), and finally a suitable quartz liquid absorption cell (f), terminating with a vent (g) to a fume hood.

[NOTE—The absorption cell will vary in optical pathlength depending on the type of mercury detection instrument used.]

![](https://example.com/image)

**Figure 6. Aeration apparatus for Mercury Limit Test**

Bottle c is fitted with an extra-coarse fritted bubbler (Corning 31770 125 EC, or equivalent), and the bottle is marked with a 60-mL calibration line. The drying tube e is lightly packed with magnesium perchlorate. Bottle c is used for the test solution, and bottle d, which remains empty throughout the procedure, is used to collect water droplets.

Alternatively, an apparatus embodying the principle of the assembly described and illustrated may be used. The aerating medium may be either compressed air or compressed nitrogen.

**Standard preparation:** Transfer 1.71 g of mercuric nitrate [Hg(NO₃)₂ · H₂O] into a 1000-mL volumetric flask, dissolve in a mixture of 100 mL of water and 2 mL of nitric acid, dilute with water to volume, and mix. Discard after 1 month. Transfer 10.0 mL of this solution into a second 1000-mL volumetric flask, acidify with 5 mL of a 1:5 sulfuric acid solution, dilute with water to volume, and mix. Discard after 1 week. For the analysis, transfer 10.0 mL of this solution into a 200-mL volumetric flask, acidify with 5 mL of a 1:5 sulfuric acid solution, and dilute with water to volume, and mix. Each mL of this solution contains 1 µg of mercury. Transfer 2.0 mL of this solution (2 µg Hg) into a 50-mL beaker, and add 20 mL of water, 1 mL of a 1:5 sulfuric acid solution, and 1 mL of a 1:25 solution of potassium permanganate. Cover the beaker with a watch glass, boil for a few seconds, and cool.

**Sample preparation:** Prepare as directed in the individual monograph.

**Procedure:** Assemble the aerating apparatus as shown in Figure 6, with bottles c and d empty and stopcock b in the bypass position. Connect the apparatus to the absorption cell (f) in the instrument, and adjust the air or nitrogen flow.
rate so that in the following procedure, maximum absorption and reproducibility are obtained without excessive foaming in the Test solution. Obtain a baseline reading at 253.6 nm, following the manufacturer’s instructions for operating the instrument.

Treat the Standard preparation as follows: destroy the excess permanganate by adding a 1:10 solution of hydroxylamine hydrochloride, dropwise, until the solution is colorless. Immediately wash the solution into bottle c with water, and dilute with water to the 60-mL mark. Add 2 mL of 10% Stannous chloride solution (prepared fresh each week by dissolving 10 g of SnCl₂·2H₂O in 20 mL of warm hydrochloric acid and diluting with 80 mL of water), and immediately reconnect bottle c to the aerating apparatus. Turn stopcock b from the bypass to the aerating position, and continue the aeration until the absorption peak has been passed and the recorder pen has returned to the baseline. Disconnect bottle c from the aerating apparatus, discard the Standard preparation mixture, wash bottle c with water, and repeat the foregoing procedure using the Sample preparation; any absorbance produced by the Sample preparation does not exceed that produced by the Standard preparation.

**Method I**

Dithizone extraction solution: Dissolve 30 mg of dithizone in 1000 mL of chloroform, add 5 mL of alcohol, and mix. Store in a refrigerator. Before use, shake a suitable volume of the solution with about half its volume of 1:100 nitric acid, discarding the nitric acid. Discard the solution after 1 month.

Diluted dithizone extraction solution: Just before use, dilute 5 mL of Dithizone extraction solution with 25 mL of chloroform.

Hydroxylamine hydrochloride solution: Dissolve 20 g of hydroxylamine hydrochloride in sufficient water to make about 65 mL, transfer the solution into a separator, add a few drops of thymol blue TS, and then add ammonium hydroxide until a yellow color develops. Add 10 mL of a 1:25 solution of sodium diethylthiocarbamate, mix, and allow to stand for 5 min. Extract the solution with successive 10- to 15-mL portions of chloroform until a 5-mL test portion of the chloroform extract does not develop a yellow color when shaken with a dilute solution of cupric sulfate. Add 2.7 N hydrochloric acid until the extracted solution is pink, adding one or two more drops of thymol blue TS, if necessary, then dilute with water to 100 mL, and mix.

Mercury stock solution: Transfer 135.4 mg of mercuric chloride, accurately weighed, into a 100-mL volumetric flask, dissolve in and dilute with 1 N sulfuric acid to volume, and mix. Dilute 5.0 mL of this solution with 1 N sulfuric acid to 500.0 mL. Each mL contains the equivalent of 10 µg of mercury.

Diluted standard mercury solution: On the day of use, transfer 10.0 mL of Mercury stock solution into a 100-mL volumetric flask, dilute with 1 N sulfuric acid to volume, and mix. Each mL contains the equivalent of 1 µg of mercury.

Sodium citrate solution: Dissolve 250 g of sodium citrate dihydrate in 1000 mL of water.

Sample solution: Dissolve 1 g of sample in 30 mL of 1.7 N nitric acid by heating on a steam bath. Cool to room temperature in an ice bath, stir, and pass through S and S No. 589, or equivalent, filter paper that has been previously washed with 1.7 N nitric acid, followed by water. Add 20 mL of Sodium citrate solution and 1 mL of Hydroxylamine hydrochloride solution to the filtrate.

Procedure: [Note—Because mercuric dithizone is light sensitive, perform this procedure in subdued light.] Prepare a control containing 3.0 mL of Diluted standard mercury solution (3 µg Hg), 30 mL of 1.7 N nitric acid, 5 mL of Sodium citrate solution, and 1 mL of Hydroxylamine hydrochloride solution. Treat the control and the Sample solution as follows: using a pH meter, adjust the pH of each solution to 1.8 with ammonium hydroxide, and transfer the solutions into different separators. Extract each with two 5-mL portions of Dithizone extraction solution, and then extract again with 5 mL of chloroform, discarding the aqueous solutions. Transfer the combined extracts from each separator into different separators, add 10 mL of 1:2 hydrochloric acid to each, shake well, and discard the chloroform layers. Extract the acid solutions with about 3 mL of chloroform, shake well, and discard the chloroform layers. Add 0.1 mL of 0.05 M disodium EDTA and 2 mL of 6 N acetic acid to each separator, mix, and then slowly add 5 mL of ammonium hydroxide. Stopper the separators, cool under a stream of cold water, and dry the outside of the separators. To avoid loss, carefully pour the solutions through the tops of the separators into separate beakers, and using a pH meter, adjust the pH of both solutions to 1.8 with 6 N ammonium hydroxide. Return the sample and control solutions to their original separators, add 5.0 mL of Diluted dithizone extraction solution, and shake vigorously. Any color developed in the Sample solution does not exceed that in the control.

**Nickel Limit Test**

[Note—Unless otherwise specified in the individual monograph, use Method I.]

**Method I**

Atomic absorption system apparatus: Use a suitable atomic absorption spectrometer equipped with a nickel hollow-cathode lamp and an air–acetylene flame to measure the absorbance of the Blank preparation, the Standard preparations, and the Test preparation as directed under Procedure.

Test preparation: Dissolve 20.0 g of sample in dilute acetic acid TS, and dilute with the same solvent to 150.0 mL. Add 2.0 mL of a saturated solution of ammonium pyrrolidinedithiocarbamate (about 10 g/L of water) and 10.0 mL of methyl isobutyl ketone, and shake for 30 s. Protect from bright light. Allow the two layers to separate, and use the methyl isobutyl ketone layer.

Blank preparation: Prepare in the same manner as in the Test preparation, but omit the sample.

Standard preparations: Prepare three Standard preparations in the same manner as in the Test preparation, but add 0.5 mL, 1.0 mL, and 1.5 mL, respectively, of 10 mg/kg nickel standard solution TS in addition to 20.0 g of sample.

Procedure: Zero the instrument with the Blank preparation. Concomitantly determine the absorbances of each of the Standard preparations and of the Test preparation at least three times each, and record the average of the steady readings for each. Between each measurement, aspirate the Blank preparation, and ascertain that the reading returns to its initial blank value.

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Method

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Calculation: Calculate the linear equation of the graph using a least-squares fit, and derive from it the concentration of nickel in the Test preparation. Alternatively, plot on a graph the mean of the readings against the added quantity of nickel. Extrapolate the line joining the points on the graph until it meets the concentration axis. The distance between this point and the intersection of the axes represents the concentration of nickel in the Test preparation.

Method II

[Note—All glassware used must be soaked in 1% nitric acid for at least 2 h, and then rinsed with water.]

1% nitric acid: Cautiously add 10 mL of nitric acid to a 1000-mL volumetric flask containing about 500 mL of water. Mix, and dilute with water to volume.

Blank solution: Use 1% nitric acid.

Nickel stock standard solution: Immediately before use, dilute an appropriate amount of nickel standard with 1% nitric acid to prepare a solution containing the equivalent of 10 µg of nickel per mL.

Standard solutions: Into three identical 100-mL volumetric flasks, introduce respectively 2.0 mL, 5.0 mL, and 10.0 mL of Nickel stock standard solution. Dilute with 1% nitric acid to volume, and mix. These standards contain 0.2 µg, 0.5 µg, and 1.0 µg of nickel per mL.

Test solution: Weigh accurately a quantity of test specimen containing about 5 g of solids into a 100-mL volumetric flask. Dissolve in and dilute with 1% nitric acid to volume, and mix.

Procedure: Concomitantly determine the absorbances of the Standard solutions and the Test solution at least three times each, at the wavelength of maximum absorbance at 232.0 nm, with a suitable atomic absorption spectrophotometer equipped with an air–acetylene flame and a nickel hollow-cathode lamp using the Blank solution to zero the instrument. Record the average of the steady readings for each of the Standard solutions and the Test solution. Clear the nebulizer using the Blank solution and aspirate each of the Standard solutions and the Test solution in turn. The standard chosen for reslope should be run every 4 to 5 samples. If there is a significant change in its response, reslope and repeat the previous samples. The standard deviation for the Standard solution of 0.2 µg of nickel per mL must be less than 20%. Plot the absorbances of the Standard solutions versus the concentration, in µg/mL, of nickel, and draw the straight line best fitting the three plotted points. From the graph so obtained, determine the concentration, C, in µg/mL, of nickel in the Test solution. Calculate the quantity, in µg, of nickel in each g of test specimen taken:

\[
\text{Result} = \frac{100C}{W}
\]
in which W is the weight, in g, of test specimen taken to prepare the Test solution.

Phosphorus Limit Test

Reagents

Ammonium molybdate solution (5%): Dissolve 50 g of ammonium molybdate tetrahydrate, \((\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}\), in 900 mL of warm water, cool to room temperature, dilute with water to 1000 mL, and mix.

Ammonium vanadate solution (0.25%): Dissolve 2.5 g of ammonium metavanadate, \(\text{NH}_4\text{VO}_3\), in 600 mL of boiling water, cool to 60°–70°, and add 20 mL of nitric acid. Cool to room temperature, dilute with water to 1000 mL, and mix.

Zinc acetate solution (10%): Dissolve 120 g of zinc acetate dihydrate, \(\text{Zn(C}_2\text{H}_4\text{O}_2\text{)}_2 \cdot 2\text{H}_2\text{O}\), in 880 mL of water, and pass through Whatman No. 2V or equivalent filter paper before use.

Nitric acid solution (29%): Add 300 mL of nitric acid (sp. gr. 1.42) to 600 mL of water, and mix.

Standard phosphorus solution (100 µg P in 1 mL): Dissolve 438.7 mg of monobasic potassium phosphate, \(\text{KH}_2\text{PO}_4\), in water in a 1000-mL volumetric flask, dilute with water to volume, and mix.

Standard curve: Pipet 5.0 mL, 10.0 mL, and 15.0 mL of the Standard phosphorus solution into separate 100-mL volumetric flasks. To each of these flasks, and to a fourth, blank flask, add in the order stated 10 mL of Nitric acid solution, 10 mL of Ammonium vanadate solution, and 10 mL of Ammonium molybdate solution, mixing thoroughly after each addition. Dilute with water to volume, mix, and allow to stand for 10 min. Determine the absorbance of each standard solution in a 1-cm cell at 460 nm, with a suitable spectrophotometer, using the blank to set the instrument to zero. Prepare a standard curve by plotting the absorbance of each solution versus its concentration, in mg of phosphorus (P) per 100 mL.

Treated sample: Place 20–25 g of the starch sample in a 250-mL beaker, add 200 mL of a 7:3 mixture of methanol and water, disperse the sample, and agitate mechanically for 15 min. Recover the starch by vacuum filtration in a 150-mL medium-porosity fritted-glass or Büchner funnel, and wash the wet cake with 200 mL of the methanol and water mixture. Reslurry the wet cake in the solvent, and wash it a second time in the same manner. Dry the filter cake in an air oven at a temperature below 50°, then grind the sample to 20-mesh or finer, and blend thoroughly. Determine the amount of dry substance by drying a 5-g portion in a vacuum oven, not exceeding 100 mm Hg, at 120° for 5 h.

[Note—The treatment outlined above is satisfactory for starch products that are insoluble in cold water. For pregelatinized starch and other water-soluble starches, prepare a 1%–2% aqueous paste, place it in a cellophane tube, and dialyze against running distilled water for 30–40 h. Precipitate the starch by pouring the solution into 4 volumes of acetone per volume of paste while stirring. Recover the starch by vacuum filtration in a medium-porosity fritted-glass or Büchner funnel, and wash the filter cake with absolute ethanol. Dry the filter cake, and determine the amount of dry substance as directed for water-insoluble starches.]

Sample preparation: Transfer about 10 g of the Treated sample, calculated on the dry-substance basis and accurately weighed, into a Vycor dish, and add 10 mL of Zinc acetate solution in a fine stream, distributing the solution uniformly in the sample. Carefully evaporate to dryness on a hot plate, then increase the heat, and carbonize the sample on the

---

4 Suitable nickel standards are available from e.g. Fisher Scientific, Fair Lawn, NJ (nickel, reference standard solution, 1000 ppm ± 1%, certified, application: for atomic absorption) or RICCA Chemical Company, Arlington, TX (nickel standard, 1000 ppm Ni, for atomic absorption).
C. OTHERS

ALGINATES ASSAY
In a suitable closed system, liberate the carbon dioxide from the uronic acid groups of about 250 mg of the test sample by heating with hydrochloric acid, and sweep the carbon dioxide, by means of an inert gas, into a titration vessel containing excess standardized sodium hydroxide. Any suitable system may be used as long as it provides precautions against leakage.
and overheating of the reaction mixture, adequate sweeping time, avoidance of entrainment of hydrochloric acid, and meets the requirements of the System Suitability Test. One suitable system, with accompanying procedure, is given below.

**Apparatus:** The apparatus is shown in Figure 7. It consists essentially of a soda lime column, \( A \), a mercury valve, \( B \), connected through a side arm, \( C \), to a reaction flask, \( D \), by means of a rubber connection. Flask \( D \) is a 100-mL round-bottom, long-neck boiling flask, resting in a suitable heating mantle, \( E \).

![Figure 7. Apparatus for Alginates Assay](image)

The reaction flask is provided with a reflux condenser, \( F \), to which is fitted a delivery tube, \( G \), of 40-mL capacity, having a stopcock, \( H \). The reflux condenser terminates in a trap, \( I \), containing 25 g of 20-mesh zinc or tin, which can be connected with an absorption tower, \( J \). The absorption tower consists of a 45-cm tube fitted with a medium-porosity fritted glass disk sealed to the inner part above the side arm and having a delivery tube sealed to it extending down to the end of the tube. A trap, consisting of a bulb of approximately 100-mL capacity, is blown above the fritted disk and the outer portion of a ground spherical joint is sealed on above the bulb. A 250-mL Erlenmeyer flask, \( K \), is connected to the bottom of the absorption tower. The top of the tower is connected to a soda lime tower, \( L \), which is connected to a suitable pump to provide vacuum and air supply, the choice of which is made by a three-way stopcock, \( M \). The volume of air or vacuum is controlled by a capillary-tube regulator or needle valve, \( N \).

All joints are a size 35/25 ground spherical type.

**Standard D-glucurono-6,3-lactone:** This chemical \( \text{C}_{6}\text{H}_{8}\text{O}_{6} \) is available as a reference standard with an assay of 100.0 ± 1.0% \((24.99 ± 0.25\% \text{ CO}_{2})\) from Aldrich Chemical Co.

**System suitability test:** Transfer about 250.0 mg of Standard D-glucurono-6,3-lactone, accurately weighed, into the reaction flask, \( D \), and carry out the Procedure described below. The system is considered suitable when the net titration results in a calculation of \% CO\(_2\) in a range of 24.73–25.26, which is equivalent to a range of 98.95%–101.06% D-glucurono-6,3-lactone.

**Procedure:** Transfer about 250 mg of sample, accurately weighed, into the reaction flask, \( D \), add 25 mL of 0.1 N hydrochloric acid, insert several boiling chips, and connect the flask to the reflux condenser, \( F \), using syrupy phosphoric acid as a lubricant.

[NOTE—Stopcock grease may be used for the other connections.]

Check the system for air leaks by forcing mercury up into the inner tube of the mercury valve, \( B \), to a height of about 5 cm. Turn off the pressure using the stopcock, \( M \). If the mercury level does not fall appreciably after 1–2 min, the apparatus may be considered to be free from leaks. Draw carbon dioxide-free air through the apparatus at a rate of 3000–6000 mL/h. Raise the heating mantle, \( E \), to the flask, heat the sample to boiling, and boil gently for 2 min. Turn off and lower the mantle, and allow the sample to cool for 15 min. Charge the delivery tube, \( G \), with 23 mL of hydrochloric acid. Disconnect the absorption tower, \( J \), rapidly transfer 25.0 mL of 0.25 N sodium hydroxide into the tower, add 5 drops of \( n \)-butanol, and reconnect the absorption tower. Draw carbon dioxide-free air through the apparatus at the rate of about 2000 mL/h, add the hydrochloric acid to the reaction flask through the delivery tube, raise the heating mantle, and heat...
the reaction mixture to boiling. After 2 h, discontinue the current of air and heating. Force the sodium hydroxide solution down into the flask, \( K \), using gentle air pressure, and then rinse down the absorption tower with three 15-mL portions of water, forcing each washing into the flask with air pressure. Remove the flask, and add to it 10 mL of a 10% solution of barium chloride (\( \text{BaCl}_2 \cdot 2\text{H}_2\text{O} \)). Stopper the flask, shake gently for about 2 min, add phenolphthalein TS, and titrate with 0.1 N hydrochloric acid. Perform a blank determination (see General Provisions). Each mL of 0.25 N sodium hydroxide consumed is equivalent to 5.5 mg of carbon dioxide (\( \text{CO}_2 \)). Calculate the results on the dried basis.

\( \alpha \)-AMINO NITROGEN (AN) DETERMINATION
Transfer 7–25 g of sample, accurately weighed, into a 500-mL volumetric flask with the aid of several 50-mL portions of warm, ammonia-free water, dilute with water to volume, and mix. Neutralize 20.0 mL of the solution with 0.2 N barium hydroxide or 0.2 N sodium hydroxide, using phenolphthalein TS as the indicator, and add 10 mL of freshly prepared phenolphthalein–formol solution (50 mL of 40% formaldehyde containing 1 mL of 0.05% phenolphthalein in 50% alcohol neutralized exactly to pH 7 with 0.2 N barium hydroxide or 0.2 N sodium hydroxide). Titrate with 0.2 N barium hydroxide or 0.2 N sodium hydroxide to a distinct red color, add a small, but accurately measured, volume of 0.2 N barium hydroxide or 0.2 N sodium hydroxide in excess, and back titrate to neutrality with 0.2 N hydrochloric acid. Conduct a blank titration using the same reagents, with 20 mL of water in place of the test solution. Each mL of 0.2 N barium hydroxide or 0.2 N sodium hydroxide is equivalent to 2.8 mg of \( \alpha \)-amino nitrogen.

AMMONIA NITROGEN (\( \text{NH}_3 \)-N) DETERMINATION
[\text{CAUTION}—Provide adequate ventilation.]
[\text{NOTE}—Use nitrogen-free reagents, where available, or reagents very low in nitrogen content.]
Transfer between 700 mg and 2.2 g of sample into a 500- to 800-mL Kjeldahl digestion flask of hard, moderately thick, well-annealed glass. If desired, wrap the sample, if solid or semisolid, in nitrogen-free filter paper to facilitate the transfer. Add about 200 mL of water, and mix. Add a few granules of zinc to prevent bumping, tilt the flask, and cautiously pour sodium hydroxide pellets, or a 2:5 sodium hydroxide solution, down the inside of the flask so that it forms a layer under the solution, using a sufficient amount (usually about 25 g of solid sodium hydroxide) to make the mixture strongly alkaline. Immediately connect the flask to a distillation apparatus consisting of a Kjeldahl connecting bulb and a condenser that has a delivery tube extending well beneath the surface of a measured excess of 0.5 N hydrochloric or sulfuric acid contained in a 500-mL flask. Add 5 to 7 drops of methyl red indicator (1 g of methyl red in 200 mL of alcohol) to the receiver flask. Rotate the Kjeldahl flask to mix its contents thoroughly, and heat until all of the ammonia has distilled, collecting at least 150 mL of distillate. Wash the tip of the delivery tube, collecting the washings in the receiving flask, and titrate the excess acid with 0.5 N sodium hydroxide. Perform a blank determination (see General Provisions), substituting 2 g of sucrose for the sample, and make any necessary correction. Each mL of 0.5 N acid consumed is equivalent to 7.003 mg of ammonia nitrogen.

[\text{NOTE}—If it is known that the substance to be determined has a low nitrogen content, 0.1 N acid and alkali may be used, in which case each mL of 0.1 N acid consumed is equivalent to 1.401 mg of nitrogen.]
Calculate the percent ammonia nitrogen:

\[
\text{Result} = (\text{NH}_3\text{-N}/\text{S}) \times 100
\]

in which \( \text{NH}_3\text{-N} \) is the weight, in mg, of ammonia nitrogen, and S is the weight, in mg, of the sample.

BENZENE (in Paraffinic Hydrocarbon Solvents)
\text{Apparatus:} (See Chromatography, Appendix IIA.) Use a suitable gas chromatograph, equipped with a column, or equivalent, that will elute \( n \)-decane before benzene under the conditions of the System Suitability Test. Column materials and conditions that have been found suitable for this method are listed in the accompanying tables. See Figure 8 for a typical chromatogram obtained with column No. 5.
**Reagents**

- **Isooctane**: 99 mole percent minimum containing less than 0.05 mole percent aromatic material
- **Benzene**: 99.5 mole percent minimum
- **Internal standard**: n-Decane and either n-undecane or n-dodecane according to the requirement of the System suitability test
- **Reference solution A**: Prepare a standard solution containing 0.5% by weight each of the Internal standard and of benzene in isooctane.
- **Reference solution B**: Prepare a standard solution containing about 0.5% by weight each of n-decane, of Internal standard, and of benzene in isooctane.

**Calibration**: Select the instrument conditions necessary to give the desired sensitivity. Inject a known volume of Reference solution A, and change the attenuation, if necessary, so that the benzene peak is measured with a chart deflection of not less than 25% or more than 95% of full scale. When choosing the attenuation, consider all unresolved peaks to represent a single compound. There may be tailing of the nonaromatic peak, but do not use any conditions that lead to a depth of the valley ahead of the benzene peak (A) less than 50% of the weight of the benzene peak (B) as depicted in Figure 9.

If there is tailing of the nonaromatic material, construct a baseline by drawing a line from the bottom of the valley ahead of the benzene peak to the point of tangency after the peak (see Figure 10). Measure the areas of the benzene peak and the internal standard peak by any of the following means: triangulation, planimeter, paper cutout, or mechanical or electronic integrator. Do not use integrators on peaks without a constant baseline, unless the integrator has provision for making baseline corrections with accuracy at least as good as that of manual methods.
Figure 10. Illustration of A/B Ratio for a Small Component Peak on the Tail of a Large Peak

Calculate a response factor for benzene \( (R_b) \) relative to the \textit{Internal standard}:

\[
\text{Result} = \frac{A_i}{W_i} \times \frac{W_b}{A_b}
\]

in which \( A_i \) is the area of the \textit{Internal standard} peak in arbitrary units corrected for attenuation; \( W_i \) is the weight percent of \textit{Internal standard} in \textit{Reference solution A}; \( W_b \) is the weight percent of benzene in \textit{Reference solution A}; and \( A_b \) is the area of the benzene peak in arbitrary units corrected for attenuation.

**Procedure:** Place approximately 0.1 mL of \textit{Internal standard} into a tared 25-mL volumetric flask, weigh on an analytical balance, and dissolve in and dilute with the sample to be analyzed to volume.

Using the exact instrumental conditions that were used in the calibration, inject the same volume of sample containing the \textit{Internal standard}. Before measuring the area of the \textit{Internal standard} and benzene peaks, change the attenuation to ensure at least 25% chart deflection.

Measure the area of the \textit{Internal standard} and benzene peaks in the same manner as was used for the calibration. Calculate the weight percentage of benzene in the sample \( (W_B) \):

\[
\text{Result} = \frac{(A_b \times R_b \times W_i \times 100)}{(A_i \times S)}
\]

in which \( A_b \) is the area of the benzene peak corrected for attenuation; \( R_b \) is the relative response factor for benzene; \( W_i \) is the weight, in grams, of \textit{Internal standard} added; \( A_i \) is the area of the \textit{Internal standard} peak corrected for attenuation; and \( S \) is the weight, in grams, of the sample taken.

**System suitability test:** Inject the same volume of \textit{Reference solution B} as in the \textit{Calibration} and record the chromatogram. \textit{n-Decane} must be eluted before benzene, and the ratio of \( A \) to \( B \) (Figure 9) must be at least 0.5 where \( A \) is equal to the depth of the valley between the \textit{n}-decane and benzene peaks and \( B \) is equal to the height of the benzene peak.

<table>
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Published on March 26, 2020
Column Materials and Conditions for the Determination of Benzene in Hexanes (continued)

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<td>El</td>
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<td>Tri</td>
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Abbreviations Used in Table: AW—Acid washed; CEF—N,N-Bis(2-cyanoethyl)formamide; DEGS—Diethylene Glycol Succinate; DI—Disk integrator; EI—Electronic integrator; FI—Flame ionization; Sil—Silanized; TC—Thermal conductivity; TCEPE—Tetracyanoethylated Pentaerythritol; Tri—Triangulation.

Retention Times in Minutes for Selected Hydrocarbons under the Conditions for the Determination of Benzene in Hexanes

<table>
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<th>Column No.</th>
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<td>o-Xylene</td>
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<td>n-Dodecane</td>
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<td>12.8</td>
<td>8.5</td>
<td>6.5</td>
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Color Determination

Chromium

Standards

Standard chromium solution (1000 mg/kg): Transfer 2.829 g of K$_2$Cr$_2$O$_7$, accurately weighed (NIST No. 136), into a 1-L volumetric flask; dissolve in and dilute with water to volume.

Standard colorant solution: Transfer 62.5 g of colorant previously shown to be free of chromium to a 1-L volumetric flask; dissolve in and dilute with water to volume.

Apparatus: Use any suitable atomic absorption spectrophotometer equipped with a fast response recorder and capable of measuring the radiation absorbed at 357.9 nm.

Instrument parameters: Wavelength setting: 357.9 nm; optical passes: 5; lamp current: 8 mA; lamp voltage: 500 v; fuel: hydrogen; oxidant: air; recorder: 1 mv with a scale expansion of 5 or 10. Alternatively, follow the instructions supplied with the instrument.

Procedure: Set the instrument at the optimum conditions for measuring chromium as directed by the manufacturer's instructions. Prepare a series of seven standard chromium solutions containing Cr at approximately 5 mg/kg, 10 mg/kg, 15 mg/kg, 20 mg/kg, 40 mg/kg, 50 mg/kg, and 60 mg/kg by appropriate dilutions of the Standard chromium solution into 100-mL volumetric flasks; add 80 mL of the Standard colorant solution, and dilute each flask with water to volume.

Transfer 5 g of the colorant to be analyzed to a 100-mL volumetric flask; dissolve in and dilute with water to volume. Prepare a calibration curve using the series of standards, and using this curve, determine the chromium content of the colorant samples.

Ether extracts

[CAUTION—Isopropyl ether forms explosive peroxides. To ensure the absence of peroxides, perform the following test: prepare a colorless solution of ferrous thiocyanate by mixing equal volumes of 0.1 N ferrous sulfate and 0.1 N ammonium thiocyanate. Using titanous chloride, carefully discharge any red coloration due to ferric ions. Add 10 mL of ether to 50 mL of the solution, and shake vigorously for 2–3 min. A red color indicates the presence of peroxides. If redistillation is necessary, the usual precautions against peroxide detonation should be observed. Immediately before use, pass the ether through a 30-cm column of chromatography-grade aluminum oxide to remove peroxides and inhibitors.]

Apparatus: Use an upward displacement-type liquid–liquid extractor, as shown in Figure 11, with a sintered-glass diffuser and a working capacity of 200 mL. Suspend a piece of bright copper wire through the condenser, and place a small coil of copper wire (about 0.5 g) in the distillation flask.

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5 To be used or sold for use to color food that is marketed in the United States, color additives must be from batches that have been certified by the U.S. Food and Drug Administration (FDA). If color additives are not from FDA-certified batches, they are not permitted color additives for food use in the United States, even if they are compositionally equivalent. The FD&C names can be applied only to FDA-certified batches of these color additives.
Alkaline ether extract: Transfer 5 g of the colorant to a beaker, and dissolve in 150 mL of water. Add 2 mL of 2.5 N NaOH solution, transfer the solution into the extractor, and dilute with water to approximately 200 mL. Add 200 mL of ether to the distillation flask, and extract for 2 h with a reflux rate of about 15 mL/min. Set the extracted colorant solution aside. Transfer the ether extract into a separatory funnel, and wash with two 25-mL portions of 0.1 N NaOH followed by two 25-mL portions of water. Reduce the volume of the ether extract to about 5 mL by distillation (in portions) from a tared flask containing a small piece of clean copper coil.

Acid ether extract: Add 5 mL of 3 N hydrochloric acid to the extracted colorant solution set aside in the alkaline ether extract procedure above, mix, and extract with ether as directed above. Wash the ether extract with two 25-mL portions of 0.1 N hydrochloric acid and water. Transfer the washed ether in portions to the flask containing the evaporated alkaline extract, and carefully remove all the ether by distillation. Dry the residue in an oven at 85° for 20 min. Then allow the flask to cool in a desiccator for 30 min, and weigh. Repeat drying and cooling until a constant weight is obtained. The increase in weight of the tared flask, expressed as a percentage of the sample weight, is the combined ether extract.

Leuco base

Reagents and solutions

Cupric chloride solution: Transfer 10.0 g of CuCl₂ · 2H₂O to a 1-L volumetric flask; dissolve in and dilute with dimethylformamide (DMF) to volume.

Sample solution: Prepare as directed in the individual monograph.

Procedure

Solution 1: Pipet 50 mL of DMF into a 250-mL volumetric flask, cover, and place in the dark.

Solution 2: Pipet 10 mL of the Sample solution into a 250-mL volumetric flask, add 50 mL of DMF, and place in the dark.

Solution 3: Pipet 50 mL of Cupric chloride solution into a 250-mL volumetric flask, and gently bubble air through the solution for 30 min.

Solutions 4a and 4b: Pipet 10 mL of the Sample solution into each of two 250-mL volumetric flasks, add 50 mL of Cupric chloride solution to each, and bubble air gently through the solutions for 30 min.

Dilute all of the solutions with water nearly to volume; incubate for 5–10 min, but no longer, in a water bath cooled with tap water; and dilute to volume. Record the spectrum for each solution between 500 nm and 700 nm using an absorbance range of 0 to 1 and a 1-cm pathlength cell; record all spectra on the same spectrogram.
<table>
<thead>
<tr>
<th>Curve No.</th>
<th>Solution in Sample Cell</th>
<th>Solution in Reference Cell</th>
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</thead>
<tbody>
<tr>
<td>I</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>II</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>III</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>IVa</td>
<td>3</td>
<td>4a</td>
</tr>
<tr>
<td>IVb</td>
<td>3</td>
<td>4b</td>
</tr>
</tbody>
</table>

**Calculation**

\[
\text{\% Leuco Base} = \frac{[(IV - III) - (II - I)] \times 2500}{a \times W \times r}
\]

in which the Roman numerals I through IV represent the absorbance readings for solutions of the corresponding Arabic numerals (above) at the wavelength maximum; \(a\) is the absorptivity (for Fast Green, \(a = 0.156\) at 625 nm; for Brilliant Blue, \(a = 0.164\) at 630 nm); \(W\) is the weight, in grams, of the sample taken; and \(r\) is the ratio of the molecular weights of colorant and leuco base (for Fast Green, \(r = 0.9712\); for Brilliant Blue, \(r = 0.9706\)).

**Mercury**

**Apparatus:** The apparatus used for the direct microdetermination of mercury is shown in Figure 12. It consists of a quartz combustion tube designed to hold a porcelain combustion boat (60 × 10 × 8 mm) and a small piece of copper oxide wire. The combustion tube is placed in a heavy-duty hinged combustion tube furnace (Lindburg Type 70T, or equivalent), and it is connected by clamped ball-joints at one end to a source of nitrogen and connected to a series of three traps at the other. The traps are constructed of a linear array of 18- × 2-mm Pyrex tubes connected by clamped ball-joints and extend from the connection at the combustion tube. Trap I contains anhydrous calcium sulfate packed between quartz-wool plugs, trap II contains ascarite packed between cotton plugs, and trap III contains aluminum oxide packed between cotton plugs. The nitrogen flow forces the mercury through the combustion tube, the three traps, and a section of Tygon tube to a mercury vapor meter (Beckman model K-23, or equivalent). The mercury released from a sample during combustion is quantitated by comparing the recorder response with that given by a series of mercury standards.

![Figure 12.](image)

Figure 12. (a) Schematic Diagram of Apparatus for Photometric Mercury Vapor M Method: A. Tank of nitrogen; B. Two-stage pressure regulator; C. Low-pressure regulator; D. Flowmeter; E. Combustion tube; F. Combustion-tube furnace; G. Dehydrite trap; H. Ascarite trap; I. Aluminum oxide trap; J. Mercury vapor meter; K. Atenuator; L. Recorder

![Figure 12 (b)](image)

Figure 12 (b) Quartz Combustion Tube with Boat and Copper Oxide Packing
Reagents and equipment

Absorbent cotton
Aluminum oxide: Anhydrous
Calcium sulfate: Anhydrous, dehydrate, or equivalent
Asbestos pads: (1 × 0.5 × 1 cm) Preheated at 800° for 1 h
Ascarite: 20- to 30-mesh
Copper oxide wire: Preheated at 850° for 2 h
Nitrogen: Purified grade
Quartz wool
Sodium carbonate: Anhydrous, fine granular

Standard solution: Transfer approximately 1.35 g of reagent-grade mercurous chloride, accurately weighed, into a 1-L volumetric flask. Dissolve in and dilute with water to volume. When diluted 100-fold, the solution contains 0.01 µg Hg per microliter (Diluted standard solution).

Procedure: Preheat the furnace to 650°, and adjust the nitrogen flow to 1 L/min.

Blank analysis: Place a square piece of preheated asbestos pad in the combustion boat, and cover it with sodium carbonate. Stop the nitrogen flow, disconnect the ball-joint, quickly insert the boat into the combustion tube with large forceps, and reconnect the joint. Note the time, allow the boat to sit in the tube with no nitrogen flow for exactly 1 min, and then restart the flow of nitrogen. Mercury elutes almost immediately with the reinstated nitrogen flow; note the recorder response. Allow about 30 s between runs.

Calibration: Determine the recorder response after the application to the asbestos pad of 1 µL, 2 µL, and 3 µL of the Diluted standard solution.

Sample analysis: Transfer 25 mg of colorant, accurately weighed, to the combustion boat, and cover the sample completely with sodium carbonate. Follow the procedure used for the Blank analysis, and calculate the mercury content using the standard curve.

Trap problems

1. Some colorants (e.g., Brilliant Blue and Fast Green) may give a response that is symmetrically dissimilar to the Hg peak. If such a response “carries over” to the next sample, then the aluminum oxide trap may need to be changed.

2. If the recorder response is of inadequate sensitivity (peak height induced by 0.01 µg less than 0.5 cm), then the traps are packed too tightly. Remove or redistribute packing first in the aluminum oxide trap, then try the other traps.

3. The traps will need changing periodically as indicated by a change in the physical appearance of the trap material or by chart responses of different retention times or different symmetry from that of mercury standards.

4. If two or more standards are run in succession, a later sample might give an erroneous mercury response. Run blanks and then repeat the sample analysis to confirm the validity of the response.

Sodium chloride

Dissolve approximately 2 g of colorant, accurately weighed, in 100 mL of water, and add 10 g of activated carbon that is free of chloride and sulfate. Boil gently for 2–3 min. Cool to room temperature, add 1 mL of 6 N nitric acid, and stir. Dilute with water to volume in a 200-mL volumetric flask, and then filter through dry paper. Repeat the treatment with 2-g portions of carbon until no color is adsorbed onto filter paper dipped into the filtrate.

Transfer 50 mL of filtrate to a 250-mL flask. Add 2 mL of 6 N nitric acid, 5 mL of nitrobenzene, and 10 mL of standardized 0.1 N silver nitrate solution. Shake the flask until the silver chloride coagulates. Prepare a saturated solution of ferric ammonium sulfate, and add just enough concentrated nitric acid to discharge the red color; add 1 mL of this solution to the 250-mL flask to serve as the indicator. Titrate with 0.1 N ammonium thiocyanate solution that has been standardized against the silver nitrate solution until the color persists after shaking for 1 min. Calculate the weight percent of sodium chloride, P:

\[
P = [(V \times N) / W] \times 22.79
\]

in which V is the net volume, in mL, of silver nitrate solution required; N is the normality of the silver nitrate solution; and W is the weight, in grams, of the sample taken. The factor 22.79 incorporates a total volume of 195 mL because 10 g of activated carbon occupies 5 mL.

Sodium sulfate

Place 25 mL of the decolorized filtrate obtained from the Sodium Chloride test into a 125-mL Erlenmeyer flask, and add 1 drop of a 0.5% phenolphthalein solution in 50% ethanol. Add 0.05 N sodium hydroxide, dropwise, until the solution is alkaline to pH paper, and then add 0.002 N hydrochloric acid until the indicator is decolorized. Add
25 mL of ethanol and about 0.2 g of tetrahydroxyquinone indicator (CAS: 319-89-1). Titrate with 0.03 N barium chloride solution to a red endpoint. Make a blank determination.

Calculate the weight percent, P, of sodium sulfate:

\[ P = \left[ (V - B) \times \frac{N}{W} \right] \times 55.4 \]

in which \( V \) is the volume, in mL, of barium chloride solution required to titrate the sample; \( B \) is the volume, in mL, of barium chloride solution required for the blank; \( N \) is the normality of the barium chloride solution; and \( W \) is the weight, in grams, of the sample taken. The factor 55.4 incorporates a total volume of 195 mL because 10 g of activated carbon occupies 5 mL.

**Total color**

**Method I (Spectrophotometric)**

Pipet 10.0 mL of the dissolved colorant into a 250-mL Erlenmeyer flask containing 90 mL of 0.04 N ammonium acetate, and mix well. Determine the net absorbance of the solution relative to water at the wavelength maximum given for each color. Calculate the percentage of colorant present:

\[ \% \text{total color} = \frac{A}{a \times C \times b} \times 100 \]

in which \( A \) is the absorbance; \( a \) is the absorptivity specified in the individual monograph (L/(mg · cm)); \( C \) is the concentration of sample in the final test solution (mg/L); and \( b \) is the cell pathlength (cm).

**Method II (Titration with Titanium Chloride)**

**Apparatus:** The apparatus for determining total color by titration with titanium chloride (TiCl\(_3\)) is shown in Figure 13. It consists of a storage bottle, \( A \), of 0.1 N titanium chloride titrant maintained under hydrogen produced by a Kipp generator; an Erlenmeyer flask, \( B \), equipped with a source of CO\(_2\) or N\(_2\) to maintain an inert atmosphere in which the reaction takes place; a stirrer; and the buret, \( C \).

---

6 >99% purity (Catalog Number 099165, Matrix Scientific, Columbia, SC, USA), or equivalent.
Reagents and solutions

Titanium chloride solution (0.1 N): Transfer 73 mL of commercially prepared 20% TiCl₃ solution into a storage bottle, and carefully add 82 mL of concentrated HCl per L of final solution. Mix well, and bubble CO₂ or N₂ through the solution for 1 h. Before standardizing, maintain the solution under a hydrogen atmosphere for at least 16 h using a Kipp generator.

Potassium dichromate solution (0.1 N, primary standard): Transfer 4.9032 g of K₂Cr₂O₇ (NIST No. 136) to a 1-L volumetric flask; dissolve in and dilute with water to volume.

Ammonium thiocyanate (50%): Transfer 500 g of NH₄SCN, ACS certified, to a 1-L volumetric flask; dissolve in about 600 mL of water, warming if necessary; and dilute to volume.

Ferrous ammonium sulfate: Fe(NH₄)₂(SO₄)₂ · 6H₂O, ACS certified

Sodium bitartrate

Standardization of the Titanium chloride solution: Drain any standing titanium chloride (TiCl₃) from the feed lines and buret, and refill with fresh solution. Add 3.0 g of Ferrous ammonium sulfate to a wide-mouth Erlenmeyer flask followed by 200 mL of water, 25 mL of 50% sulfuric acid, 25 mL of 0.1 N Potassium dichromate solution (by pipet), and 2 or 3 boiling chips. Boil the solution vigorously on a hot plate for 30 s to remove dissolved air, then quickly transfer the flask to the titration apparatus, securely connect the stopper assembly, and start the carbon dioxide flow and stirrer. Pass carbon dioxide over the solution for 1 min before beginning the titration.

Add the 0.1 N Titanium chloride solution at a fast, steady drip to within 1 mL of the estimated endpoint (about 20 mL). Reduce the carbon dioxide flow, remove the solid-glass rod from the stopper assembly, pipet 10 mL of Ammonium thiocyanate (50%) into the flask, insert the glass rod, and increase the carbon dioxide flow. Continue titrating slowly until the endpoint: a color change from brown-red to light green is observed. Perform a blank determination using the same reagents and quantities, and calculate the normality, N, of the 0.1 N Titanium chloride solution on the basis of three titrations:
METHOD

Primary eluant: 0.01 M aqueous Na\textsubscript{2}B\textsubscript{4}O\textsubscript{7}
Secondary eluant: 0.20 M NaClO\textsubscript{4} in aqueous 0.01 M Na\textsubscript{2}B\textsubscript{4}O\textsubscript{7}
Sample size: 20 \mu L of a 0.25% solution
Flow rate: 0.60 mL/min
Gradient: Linear, in two phases: 0%–18% in 40 min, 18%–62% in 8 min more, then hold for 18 min more at 62%
Temperature: 50°
Pressure: 1000 psi

Calculation: After identifying each intermediate and side product by comparing spectra of the fractions with commercial counterparts, calculate the total color, \( T \), in percent and on the basis of three titrations:

\[
T = \left( \frac{V_s - V_b}{W \times F_s} \right) \times 100 \times N
\]

in which \( V_s \) is the volume of titrant required to produce the endpoint in a blank titration; \( V_b \) is the volume, in mL, of 0.1 N Potassium dichromate used; \( N_b \) is the normality of the titrant.

\( N = \left( \frac{V_i \times N_b}{V_i - V_b} \right) \)

in which \( V_i \) is the volume, in mL, of 0.1 N Potassium dichromate used; \( N_b \) is the normality of the 0.1 N Potassium dichromate; \( V_i \) is the volume, in mL, of 0.1 N Titanium chloride solution used; and \( V_b \) is the volume, in mL, of titanium dichloride used in the blank titration.

Procedure: Transfer the quantity of colorant prescribed in the individual monograph into a 500-mL wide-mouth Erlenmeyer flask and add 21–22 g of Sodium Bitartrate (sodium citrate for Sunset Yellow), 275 mL of water, and two or three boiling chips. Boil the solution vigorously on a hot plate for 30 s to remove dissolved air, then quickly transfer the flask to the titration apparatus, secure the stopper assembly, and start the carbon dioxide flow and stirrer. Pass carbon dioxide over the solution for 1 min before beginning the titration.

Titrates the sample until the color lightens, wait 20 s, and then continue the addition with about 2 s between drops. When the color is almost completely bleached, wait 20 s, and then continue the addition with 5 s between drops. A complete color change indicates the endpoint. Perform a blank determination using the same reagents and quantities, and calculate the total color, \( T \), in percent and on the basis of three titrations:

\[
T = \left( \frac{V_s - V_b}{W \times F_s} \right) \times 100 \times N
\]

in which \( V_s \) is the volume of titrant used; \( V_b \) is the volume of titrant required to produce the endpoint in a blank; \( W \) is the weight, in grams, of the sample taken; \( F_s \) is a factor derived from the stoichiometry of the reaction characteristics of each colorant and is given in the individual monograph; and \( N \) is the normality of the titrant.

Method III (Gravimetric)

Transfer approximately 0.5 g of colorant, accurately weighed, to a 400-mL beaker, add 100 mL of water, and heat to boiling. Add 25 mL of 1:50 hydrochloric acid, and bring to a boil. Wash down the sides of the beaker with water, cover, and keep on a steam bath for several hours or overnight. Cool to room temperature, and quantitatively transfer the precipitate into a tared filtering crucible with 1:100 hydrochloric acid. Wash the precipitate with two 15-mL portions of water, and dry the crucible for 3 h at 135°. Cool in a desiccator, and weigh. Calculate the total color, \( P \), in weight percent:

\[
P = \left( \frac{W_p \times F}{W_s} \right) \times 100
\]

in which \( W_p \) is the weight, in grams, of the precipitate; \( F \) is the gravimetric conversion factor given in the individual monograph; and \( W_s \) is the original weight, in grams, of the sample taken.

Uncombined intermediates and products of side reactions

- **METHOD I**

Sample solution: Transfer approximately 2 g of colorant to a 100-mL volumetric flask; dissolve in and dilute with water to volume.

Apparatus: Pack a 2.5-cm \( \times \) 45-cm glass column with approximately 20 g of cellulose (Whatman CF-11 grade, or equivalent) that has been slurried in the eluant and from which the fines have been removed by decantation.

Equilibrate the column thoroughly with the eluant, 35% ammonium sulfate.

Procedure: Pipet 5 mL of the Sample solution into a beaker containing 5 g of cellulose that has been slurried in eluant and from which the fines have been removed by decantation. Stir the mixture thoroughly, add 10 g of ammonium sulfate, and stir until uniformly mixed. Mix the slurry with 15 mL of eluant, and apply it to the column. Allow the fluid to enter the column, and wash the beaker with eluant until the sample is quantitatively transferred. Elute the column with approximately 500 mL of 35% ammonium sulfate, and collect a total of eight 60-mL fractions. Divide each collected fraction in half and add 0.5 mL of NH\textsubscript{4}OH to one half and 0.5 mL of HCl to the other.

Calculation: After identifying each intermediate and side product by comparing spectra of the fractions with commercial standards, calculate the concentration, \( C \), of each:

\[
C = \frac{A}{a \times b}
\]

in which \( A \) is the absorbance at the wavelength of maximal absorption; \( a \) is the absorptivity given in the individual monograph; and \( b \) is the cell pathlength, in cm.

- **METHOD II**

Apparatus: Use a suitable high-performance liquid chromatography system (see Chromatography, Appendix IIA) equipped with a dual wavelength detector system such that the effluent can be monitored serially at 254 nm and 325–385 nm (wide-band pass). Use a 1-m \( \times \) 2.1-mm (id) column, or equivalent, packed with a strong anion-exchange resin (Dupont No. 830950405, or equivalent).

Operating conditions: The operating conditions required may vary depending on the system used. The following conditions have been shown to give suitable results for Allura Red, Tartrazine, and Sunset Yellow.

Allura red

<table>
<thead>
<tr>
<th>Condition</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary eluant</td>
<td>0.01 M aqueous Na\textsubscript{2}B\textsubscript{4}O\textsubscript{7}</td>
</tr>
<tr>
<td>Secondary eluant</td>
<td>0.20 M NaClO\textsubscript{4} in aqueous 0.01 M Na\textsubscript{2}B\textsubscript{4}O\textsubscript{7}</td>
</tr>
<tr>
<td>Sample size</td>
<td>20 \mu L of a 0.25% solution</td>
</tr>
<tr>
<td>Flow rate</td>
<td>0.60 mL/min</td>
</tr>
<tr>
<td>Gradient</td>
<td>Linear, in two phases: 0%–18% in 40 min, 18%–62% in 8 min more, then hold for 18 min more at 62%</td>
</tr>
<tr>
<td>Temperature</td>
<td>50°</td>
</tr>
<tr>
<td>Pressure</td>
<td>1000 psi</td>
</tr>
</tbody>
</table>

Published on March 26, 2020
Order of elution: (1) Cresidinesulfonic acid (CSA); (2) unknown; (3) Schaeffer’s salt (SS); (4) unknown; (5) 4,4′-diazooaminobis(5-methoxy-2-methylbenzenesulfonic acid) (DMMA); (6) unknown; (7) Allura Red; (8) 6,6′-oxybis(2-naphthalenesulfonic acid) (DONS)

**Tartrazine**
- **Primary eluant:** 0.01 M aqueous Na$_2$B$_4$O$_7$
- **Secondary eluant:** 0.10 M NaClO$_4$ in aqueous 0.01 M Na$_2$B$_4$O$_7$
- **Sample size:** 50 µL of a 0.15% solution, prepared within 13 min of injection
- **Flow rate:** 1.00 mL/min
- **Gradient:** Exponential at 4%/min: 0.95%
- **Temperature:** 50°
- **Pressure:** 1000 psi

Order of elution: (1) Phenylhydrazine-$p$-sulfonic acid (PHSA); (2) sulfanilic acid (SA); (3) 1-(4-sulfophenyl)-3-ethylcarboxy-5-hydroxypyrazolone (PY-T); (4) 1-(4-sulfophenyl)-3-carboxy-5-hydroxypyrazolone (EEPT); (5) 4,4′-(diazoamino)-dibenzenesulfonic acid (DAADBSA)

**Sunset yellow**
- **Primary eluant:** 0.01 M aqueous Na$_2$B$_4$O$_7$
- **Secondary eluant:** 0.20 M NaClO$_4$ in aqueous 0.01 M Na$_2$B$_4$O$_7$
- **Sample size:** 5 µL of a 1% solution
- **Flow rate:** 0.50 mL/min
- **Gradient:** Linear in four phases: 0%–11% in 10 min; hold 25 min; 11%–38% in 10 min; 38%–42% in 10 min; 42%–98% in 20 min; hold 20 min
- **Temperature:** 50°
- **Pressure:** 1000 psi

Order of elution: (1) Sulfanilic acid (SA); (2) Schaeffer’s salt (SS); (3) 4,4′-(diazoamino)-dibenzenesulfonic acid (DAADBSA); (4) $R$-salt dye; (5) Sunset Yellow; (6) 6,6′-oxybis(2-naphthalenesulfonic acid) (DONS)

**Standard solutions**
- **Allura red:** Prepare a solution containing 0.25 g of colorant, 0.5 mg of CSA, 0.75 mg of SS, 0.25 mg of DMMA, and 1.25 mg of DONS in a 100-mL volumetric flask. Dissolve in and dilute with 0.1 M Na$_2$B$_4$O$_7$ to volume.
- **Tartrazine:** Prepare a solution containing 0.15 g of colorant and 0.3 mg each of PHSA, SA, PY-T, EEPT, and DAADBSA in a 100-mL volumetric flask. Dissolve in and dilute with 0.1 M Na$_2$B$_4$O$_7$ to volume.
- **Sunset yellow:** Prepare a solution containing 0.25 g of colorant, 0.5 mg of SA, 0.75 mg of SS, 0.25 mg of DAADBSA, and 1.25 mg of DONS in a 100-mL volumetric flask. Dissolve in and dilute with 0.1 M Na$_2$B$_4$O$_7$ to volume.

**Test solutions:** Prepare at least four test solutions, each containing the colorant, and one impurity, accurately weighed, dissolved in 0.1 M Na$_2$B$_4$O$_7$, and diluted to volume in a 100-mL volumetric flask. The solutions should encompass the range of concentrations, evenly spaced, given below for each constituent:
- **Allura red (250 mg):** CSA (0.05–0.5 mg); SS (0.05–0.75 mg); DONS (0.5–2.5 mg); DMMA (0.025–0.25 mg). Inject 20 µL of each solution.
- **Tartrazine (150 mg):** SA (7.5 to 300 µg); PY-T (7.5–300 µg); EEPT (7.5–300 µg); DAADBSA (7.5–300 µg). Inject 50 µL of each solution.
- **Sunset yellow (250 mg):** SA (0.05–0.5 mg); SS (0.05–0.75 mg); DONS (0.5–2.5 mg); DAADBSA (0.05–0.25 mg). Inject 20 µL of each solution.

**System suitability**
- **Resolution:** Elute the column, or equivalent, with the gradient specified under Operating conditions until a smooth baseline is obtained. Inject an aliquot of the Standard solution. The resolution of the eluted components matches or exceeds that shown for the corresponding colorant (see Figures 14, 15, and 16). After determining that the column, or equivalent, will give the required resolution, allow it to rest for 2 weeks before use.
Figure 14. Allura Red—Top Trace: Eluant Monitored at 254 nm; Bottom Trace: Eluant Monitored at 375–385 nm

Figure 15. Tartrazine—Top Trace: Eluant Monitored at 254 nm; Bottom Trace: Eluant Monitored at 375–385 nm
Figure 16. Sunset Yellow–Top Trace: Eluant Monitored at 254 nm; Bottom Trace: Eluant Monitored at 375–385 nm

**Calibration:** Inject the designated volume of each *Test solution* onto a conditioned column, and prepare a standard curve corresponding to each unreacted intermediate and side reaction product. Determine the area, $A_i$, for each peak from the integrator if an automated system is used or by multiplying the peak height by the width at one-half the height. The peak height alone may be used for EEPT, PY-T, and DAADBSA. Calculate the concentration, $C_i$, of each intermediate or side product:

$$C_i = mA_i + b$$

in which $A_i$ is the area of its corresponding chromatographic peak. Calculate the slope, $m$, and intercept, $b$, using the following linear regression equations:

$$m = \frac{[N \Sigma C_iA_i - \Sigma C_i \Sigma A_i]/[N \Sigma A_i^2 - (\Sigma A_i)^2]}{[N \Sigma A_i^2 - (\Sigma A_i)^2]}$$

$$b = \bar{A_i} - m[\bar{C_i}]$$

in which $\bar{A}$ and $\bar{C}$ are the calculated averages of the peak areas and concentrations, respectively, used to construct the standard curve for one intermediate or side reaction product. Calculate the correlation coefficient, $r$:

$$r = \frac{\Sigma(C_i - \bar{C})(A_i - \bar{A})}{\sqrt{\Sigma(C_i - \bar{C})^2} \times \Sigma(A_i - \bar{A})^2}$$

Each time the system is calibrated, add the new data to those accumulated from previous analyses. The correlation coefficient must be 0.95–1.00 for any single experiment or from accumulated data. Recalibrate the system after every 10 determinations or 2 days, whichever occurs first.

**Sample preparation:** Prepare as directed in the individual monograph.

**Procedure:** Inject the volume of *Sample preparation* as designated in the monograph into the column. Determine the concentration of intermediates and side reaction products from the peak areas using the slope, $m$, and intercept, $b$, calculated under *Calibration*:

$$C_i = mA_i + b$$

in which $C_i$ is the concentration of the unknown in the *Sample preparation* and $A_i$ its corresponding peak area.

**Loss on drying (volatile matter)**

Transfer 1.5–2.5 g of colorant, accurately weighed, to a tared crucible. Heat in a vacuum oven at 135° for 12–15 h. Lower the pressure in the oven to −125 mm Hg, and continue heating for an additional 2 h. Cover the crucible, and allow to cool in a desiccator. Reweigh the crucible when cool. The loss of weight is defined as the volatile matter.
**METHOD I: ICP–OES**

Before the initial use of either of the procedures below, the analyst should ensure that the procedure is appropriate for the element of interest. **Method I** can be used for elemental impurities generally amenable to detection by inductively coupled plasma–atomic (optical) emission spectroscopy (ICP–OES). **Method II** can be used for elemental impurities generally amenable to detection by inductively coupled plasma–mass spectrometry (ICP–MS). If no method is specified in the individual monograph, analysts are instructed to use **Method II** (ICP–MS).

- **Reagents:** All reagents used for the preparation of the sample and standard solutions should be free of elemental impurities. Reagents should be commercial elemental stock standards that are NIST-traceable, or equivalent, at a recommended concentration of 100 µg/mL or greater; or appropriate USP Reference Standards, as either single element or multielement.
  - **Aqua regia:** Ultra pure nitric acid/hydrochloric acid (1:3) prepared as needed. (A 1%–5% solution of aqua regia is used as a rinsing solution between analyses and as calibration blanks.)

  - **Sample preparation:** Use this sample preparation procedure unless otherwise specified in the individual monograph.
    - **Sample solution:** Allow the digestion vessel containing the sample solution to cool (for mercury measurements, add an appropriate stabilizer, such as gold at about 0.1 ppm), and dilute with water to 50.0 mL.
    - **Calibration solution 1:** 2J of the element of interest in a matched matrix (acid concentrations similar to that of the Sample solution), where J is the limit for the specific elemental impurity. [NOTE—Multiple elements of interest may be included in this solution at the same concentration ratio. For mercury analysis, add an appropriate stabilizer, such as gold at about 0.1 ppm.]
    - **Calibration solution 2:** 0.1J of the element of interest in a matched matrix (acid concentrations similar to that of the Sample solution), where J is the limit for the specific elemental impurity. [NOTE—Multiple elements of interest may be included in this solution at the same concentration ratio. For mercury analysis, add an appropriate stabilizer, such as gold at about 0.1 ppm.]
    - **Check standard solution:** 1 ppm of the element of interest in a matched matrix (acid concentrations similar to that of the Sample solution). [NOTE—Multiple elements of interest may be included in this solution at the same concentration ratio. For mercury analysis, add an appropriate stabilizer, such as gold at about 0.1 ppm.]

  - **Blank:** Matched matrix (acid concentrations similar to that of the Sample solution)

  - **Elemental spectrometric system (see Plasma Spectrochemistry, Appendix IIC)**
    - **Mode:** ICP
    - **Detector:** Optical emission spectroscopy
    - **Rinse:** 5% Aqua Regia
    - **Calibration:** Two-point, using Calibration solution 1, Calibration solution 2, and Blank

  - **System suitability**
    - **Sample:** Check standard solution
    - **Suitability requirement:** The concentration determined from the resulting chromatogram differs from actual concentration by NMT 20%. [NOTE—If samples are high in mineral content, to minimize sample carryover, rinse system well (60 s) before introducing the Check Standard Solution.]

  - **Analysis:** Analyze according to manufacturer’s suggestions for program and wavelength. Calculate and report results on the basis of the original sample size.

  - **Calculation:** Upon completion of the analysis, calculate the final concentration of a given element in the test article (µg/g) from the solution element concentration (µg/mL) as follows:
    \[
    C = \left( \frac{A \times V_1}{W} \right) \times \left( \frac{V_2}{V_3} \right)
    \]
where $C$ is the concentration of the analyte, $\mu$g/g; $A$ is the instrument reading, $\mu$g/mL; $V_i$ is the volume of the initial test article preparation, mL; $W$ is the weight of the test article preparation, g; $V_2$ is the total volume of any dilution performed, mL; and $V_3$ is the volume of the aliquot of initial test article preparation used in any dilution performed, mL.

Similarly, calculate the final concentration of a given element in the test article ($\mu$g/g) from the solution element concentration (ng/mL) as follows:

$$C = \left\{ \frac{(A \times V_i)}{W} \times (1 \ \mu\text{g}/1000 \ \text{ng}) \frac{V_2}{V_3} \right\}$$

where $A$ is the instrument reading, ng/mL; and the other factors are as defined above.

**METHOD II: ICP–MS**

**Reagents:** All reagents used for the preparation of sample and standard solutions should be free of elemental impurities. Reagents should be commercial elemental stock standards that are NIST-traceable, or equivalent, at a recommended concentration of 100 µg/mL or greater; or appropriate USP Reference Standards, as either single element or multielement.

Aqua regia: Ultra pure nitric acid/hydrochloric acid (1:3) prepared as needed. (A 1%–5% solution of aqua regia is used as a rinsing solution between analyses and as calibration blanks.)

**Sample preparation:** Proceed as directed under Method I.

**Sample solution:** Allow the digestion vessel containing the Sample preparation to cool, and add appropriate internal standards at appropriate concentrations (for mercury measurements, gold should be one of the internal standards). Dilute with water to 50.0 mL.

**Calibration solution 1:** Proceed as directed under Method I.

**Calibration solution 2:** Proceed as directed under Method I.

**Blank:** Matched volume (acid concentrations similar to that of the Sample solution).

**Elemental spectrometric system (see Plasma Spectrochemistry, Appendix IIC)**

Mode: ICP. [NOTE—An instrument with a cooled spray chamber is recommended.]

**Detector:** Mass spectrometer

**Rinse:** 5% Aqua regia

**Calibration:** Calibration solution 1, Calibration solution 2, and Blank

**System suitability**

Sample: Calibration solution 1

Suitability requirement: The concentration determined from the resulting chromatogram differs from actual concentration by NMT 20%. [NOTE—If samples are high in mineral content, to minimize sample carryover, rinse system well (60 s) before introducing the Check standard solution.]

Analysis: Analyze according to the manufacturer’s suggestions for the program and m/z. Calculate and report results based on the original sample size. [NOTE—Arsenic is subject to interference from argon chloride. Appropriate measures, including a sample preparation without Aqua regia, must be taken to correct for the interference, depending on instrumental capabilities.]

**Calculation:** Upon completion of the analysis, calculate the final concentration of a given element in the test article ($\mu$g/g) from the solution element concentration (µg/mL) as follows:

$$C = \left\{ \frac{(A \times V_i)}{W} \times (1 \ \mu\text{g}/1000 \ \text{ng}) \frac{V_2}{V_3} \right\}$$

Similarly, calculate the final concentration of a given element in the test article ($\mu$g/g) from the solution element concentration (ng/mL) as follows:

$$C = \left\{ \frac{(A \times V_i)}{W} \times (1 \ \mu\text{g}/1000 \ \text{ng}) \frac{V_2}{V_3} \right\}$$

where $A$ is the instrument reading, ng/mL; and the other factors are as defined above.

**GLUTAMIC ACID**

**Apparatus:** Use an ion-exchange amino acid analyzer, equipped with sulfonated polystyrene columns, in which the effluent from the sample is mixed with ninhydrin reagent and the absorbance of the resultant color is measured continuously and automatically at 570 nm and 440 nm by a recording photometer.

**Standard solution:** Transfer 1.250 ± 2 mg of reagent-grade glutamic acid, accurately weighed, into a 500-mL volumetric flask. Fill the flask half-full with water; add 5 mL of hydrochloric acid to help dissolve the amino acid, dilute with water to volume, and mix. Prepare the standard for analysis by diluting 1 mL of this solution with 4 mL of 0.2 N sodium citrate, pH 2.2, buffer. This Standard solution contains 0.5 mg of glutamic acid per mL (C$_0$).

**Sample preparation:** Dilute 5 mg of sample, accurately weighed, to exactly 5 mL with 0.2 N sodium citrate, pH 2.2, buffer. Remove any insoluble material by centrifugation or filtration.

**Procedure:** Using 2-mL aliquots of the Standard solution and Sample preparation, proceed according to the apparatus manufacturer’s instructions. From the chromatograms thus obtained, match the retention times produced by the Standard preparation with those produced by the Sample Solution, and identify the peak produced by glutamic acid. Record the area of the glutamic acid peak from the sample as $A_w$ and that from the standards as $A_S$.

**Calculations:** Calculate the concentration, $C_w$, in mg/mL, of glutamic acid in the Sample preparation:

$$\text{Result} = A_w \times C_S/A_S$$
in which $C_S$ is the concentration, in mg/mL, of glutamic acid in the Standard solution.

Calculate the percent glutamic acid, on the basis of total protein:

$$\text{Result} = \frac{100 \times C_A}{(6.25 \times N_T)}$$

in which 6.25 is the conversion factor for protein and amino acids, and $N_T$ is the percent total nitrogen determined in the monograph Assay.

Calculate the percent glutamic acid in the sample:

$$\text{Result} = 100 \times \frac{C_A}{S_W}$$

in which $S_W$ is the weight, in mg, of the sample taken.

**HYDROXYPROPOXYL DETERMINATION**

**Apparatus:** The apparatus for hydroxypropoxyl group determination is shown in Figure 17.

![Figure 17. Apparatus for Hydroxypropoxyl Determination](image)

The boiling flask, $D$, is fitted with an aluminum foil-covered Vigreaux column, $E$, on the side arm and with a bleeder tube through the neck and to the bottom of the flask for the introduction of steam and nitrogen. A steam generator, $B$, is attached to the bleeder tube through tube $C$, and a condenser, $F$, is attached to the Vigreaux column. The boiling flask and steam generator are immersed in an oil bath, $A$, equipped with a thermoregulator such that a temperature of 155° and the desired heating rate may be maintained. The distillate is collected in a 150-mL beaker, $G$, or other suitable container.

**Procedure:** Unless otherwise directed, transfer about 100 mg of the sample, previously dried at 105° for 2 h and accurately weighed, into the boiling flask, and add 10 mL of chromium trioxide solution (60 g in 140 mL of water). Immers the steam generator and the boiling flask in the oil bath (at room temperature) to the level of the top of the chromium trioxide solution. Start cooling water through the condenser, and pass nitrogen gas through the boiling flask at the rate of one bubble per second. Starting at room temperature, raise the temperature of the oil bath to 155° over a period of not less than 30 min, and maintain this temperature until the end of the determination. Distill until 50 mL of distillate is collected. Detach the condenser from the Vigreaux column, and wash it with water, collecting the washings in the distillate container. Titrate the combined washings and distillate with 0.02 N sodium hydroxide to a pH of 7.0, using a pH meter set at the expanded scale.

[NOTE—phenolphthalein TS may be used for this titration if it is also used for all standards and blanks. ]

Record the volume, $V_a$, of the 0.02 N sodium hydroxide used. Add 500 mg of sodium bicarbonate and 10 mL of 2 N sulfuric acid, and then after evolution of carbon dioxide has ceased, add 1 g of potassium iodide. Stopper the flask, shake the mixture, and allow it to stand in the dark for 5 min. Titrate the liberated iodine with 0.02 N sodium thiosulfate to the sharp disappearance of the yellow color, confirming the endpoint by the addition of a few drops of starch TS. Record the volume of 0.02 N sodium thiosulfate required as $Y_a$.

Make several reagent blank determinations, using only the chromium trioxide solution in the above procedure. The ratio of the sodium hydroxide titration ($V_a$) to the sodium thiosulfatetion ($Y_a$), corrected for variation in normalities, will give the acidity-to-oxidizing ratio, $V_a/Y_a = K$, for the chromium trioxide carried over in the distillation. The factor $K$ should be constant for all determinations.

Make a series of blank determinations using 100 mg of methylcellulose (containing no foreign material) in place of the sample, recording the average volume of 0.02 N sodium hydroxide required as $V_m$ and the average volume of 0.02 N sodium thiosulfate required as $Y_m$.

Calculate the hydroxypropoxyl content of the sample, in mg:

$$\text{Result} = 75.0 \times \left[ N_1(V_a - V_m) - kN_2(Y_a - Y_m) \right]$$
in which \( N_1 \) is the exact normality of the 0.02 N sodium hydroxide solution, \( k = \frac{V_2 N_2}{Y_2 N_1} \), and \( N_2 \) is the exact normality of the 0.02 N sodium thiosulfate solution.

The percentage of substitution, by weight, of hydroxypropoxyl groups, determined as directed above, may be converted to molecular substitution per glucose unit by reference to Figure 18.

![Figure 18. Chart for Converting Percentage of Substitution, by Weight, of Hydroxypropoxyl Groups to Molecular Substitution per Glucose Unit](image)

**METHOXYL DETERMINATION**

**Apparatus:** The apparatus for methoxyl determination, as shown in Figure 19, consists of a boiling flask, \( A \), fitted with a capillary side arm to provide an inlet for carbon dioxide and connected to a column, \( B \), which separates aqueous hydriodic acid from the more volatile methyl iodide. After the methyl iodide passes through a suspension of aqueous red phosphorus in the scrubber trap, \( C \), it is absorbed in the bromine–acetic acid absorption tube, \( D \). The carbon dioxide is introduced from a device arranged to minimize pressure fluctuations and connected to the apparatus by a small capillary containing a small plug of cotton.

![Figure 19. Distillation Apparatus for Methoxyl Determination](image)

**Reagents**

- **Acetic potassium acetate:** Dissolve 100 g of potassium acetate in 1000 mL of a mixture consisting of 900 mL of glacial acetic acid and 100 mL of acetic anhydride.
- **Bromine–acetic acid solution:** On the day of use, dissolve 5 mL of bromine in 145 mL of the Acetic potassium acetate solution.
Hydriodic acid: Use special-grade hydriodic acid suitable for alkoxyl determinations, or purify reagent grade as follows: distill over red phosphorus in an all-glass apparatus, passing a slow stream of carbon dioxide through the apparatus until the distillation is terminated and the receiving flask has completely cooled.

[CAUTION—Use a safety shield, and conduct the distillation in a fume hood.]

Collect the colorless, or almost colorless, constant-boiling acid distilling between 126°–127°. Store the acid in a cool, dark place in small, brown, glass-stoppered bottles previously flushed with carbon dioxide and finally sealed with paraffin.

Procedure: Fill trap C half-full with a suspension of about 60 mg of red phosphorus in 100 mL of water, introduced through the funnel on tube D and the side arm that connects with the trap at C. Rinse tube D and the side arm with water, collecting the rinsings in trap C, then charge absorption tube D with 7 mL of Bromine-acetic acid solution. Place the sample, previously accurately weighed in a tared gelatin capsule, into the boiling flask A, along with a few glass beads or boiling stones, then add 6 mL of Hydriodic acid. Connect the flask to the condenser, using a few drops of the acid to seal the junction, and begin passing the carbon dioxide through the apparatus at the rate of about two bubbles per second. Heat the flask in an oil bath at 150°, continue the reaction for 40 min, and drain the contents of absorption tube D into a 500-mL Erlenmeyer flask containing 10 mL of a 1:4 solution of sodium acetate. Rinse tube D with water, collecting the rinsings in the flask, and dilute with water to about 125 mL. Discharge the red-brown color of bromine by adding formic acid, dropwise with swirling, then add 3 drops in excess. Usually a total of 12 to 15 drops of formic acid is required. Allow the flask to stand for 3 min, add 15 mL of 2 N sulfuric acid and 3 g of potassium iodide, and titrate immediately with 0.1 N sodium thiosulfate, adding starch TS near the endpoint. Perform a blank determination with the same quantities of the same reagents, including the gelatin capsule, and in the same manner, and make any necessary correction. Each mL of 0.1 N sodium thiosulfate is equivalent to 0.517 mg (517 µg) of methoxyl groups (–OCH3).

NITROGEN DETERMINATION (Kjeldahl Method)

[NOTE—All reagents should be nitrogen free, where available, or otherwise very low in nitrogen content.]

• Method I

Use this method unless otherwise directed in the individual monograph. It is not applicable to certain nitrogen-containing compounds that do not yield their entire nitrogen upon digestion with sulfuric acid.

Nitrites and nitrates absent

Unless otherwise directed, transfer 1 g of sample into a 500-mL to 800-mL Kjeldahl digestion flask of hard borosilicate, moderately thick, well-annealed glass, wrapping the sample, if solid or semisolid, in nitrogen-free filter paper to facilitate the transfer if desired. Add 10 g of powdered potassium sulfate or anhydrous sodium sulfate, 500 mg of powdered cupric sulfate, and 20 mL of sulfuric acid. Place the flask in an inclined position (about 45°), and heat gently keeping the temperature below the boiling point until frothing ceases, adding a small amount of paraffin, if necessary, to reduce frothing.

[CAUTION—The digestion should be conducted in a fume hood, or the digestion apparatus should be equipped with a fume exhaust system.]

Increase the heat until the acid boils briskly and continue the heating process until the solution clears, and then continue boiling for 30 min longer (or for 2 h for samples containing organic material). Cool, add about 150 mL of water, mix, and then cool to below 25°. Add cautiously 100 mL of a 2.5 sodium hydroxide solution, in such a manner as to cause the solution to flow down the inner side of the flask to form a layer under the acid solution, to make the mixture strongly alkaline. Add a few granules of zinc to prevent bumping, and immediately connect the flask to a distillation apparatus consisting of a Kjeldahl connecting bulb and a condenser, the delivery tube of which extends well beneath the surface of 100 mL of a 1.25 boric acid solution contained in a conical flask or a wide-mouth bottle of about 500-mL capacity. Gently, rotate the Kjeldahl flask to mix its contents thoroughly, and then heat until all of the ammonia has distilled, collecting at least 150 mL of distillate (about 80% of the contents of the flask). Wash the tip of the delivery tube, collecting the washings in the receiving flask, and titrate with 0.5 N sulfuric acid, determining the endpoint potentiometrically. Perform a blank determination, substituting 2 g of sucrose for the sample, and make any necessary correction (see General Provisions). Each mL of 0.5 N acid is equivalent to 7.003 mg of nitrogen.

[NOTE—An indicator solution can also be used to determine the titration endpoint. For example, dissolve 0.2 g of methyl red in 100 mL of 95% ethanol, 1 g of bromocresol green in 500 mL of 95% ethanol, then combine 1 part of the methyl red solution and 5 parts of the bromocresol green solution. Add 3 mL of methyl red/bromocresol green indicator solution per L of boric acid solution. Then, titrate the sample to the first trace of pink. Also, a bromocresol green–methyl red solution can be used as an alternative. To make the solution, dissolve 0.15 g of bromocresol green and 0.1 g of methyl red in 180 mL of alcohol, and dilute with water to a final volume of 200 mL.]

[NOTE—If the substance to be determined is known to have a low nitrogen content, 0.1 N acid and alkali may be used, in which case each mL of 0.1 N acid consumed is equivalent to 1.401 mg of nitrogen.]

[NOTE—Nitrogen recovery verification can be run to check for accuracy of the procedure and the equipment.]

1) Nitrogen loss: Use 0.12 g of ammonium sulfate and 0.85 g of sucrose. Add all other reagents, digest, and distill under the same conditions as for the sample. Recoveries should be NLT 99%.

2) Digestion efficiency: Use 0.16 g of lysine hydrochloride or 0.18 g of tryptophan, with 0.67 g of sucrose per flask. Add all other reagents, digest, and distill under the same conditions as for the sample. Recoveries should be NLT 98%.

Nitrites and nitrates present
[NOTE—This procedure is not applicable to liquids to or materials having a high chlorine-to-nitrate ratio.]

Unless otherwise directed, transfer a quantity of sample, accurately weighed, corresponding to about 150 mg of nitrogen into a Kjeldahl flask, and add 25 mL of 93%–98% sulfuric acid containing 1 g of salicylic acid. Mix thoroughly by shaking, and then allow to stand for 30 min or more, with frequent shaking. Add 5 g of Na₂S₂O₅ · 5H₂O (as an impalpable powder, not granules or filings), mix, then add 500 mg of powdered cupric sulfate, and proceed as directed under *Nitrates and Nitrites Absent*, beginning with “Incline the flask at an angle of about 45°”. When the nitrogen content of the substance is known to exceed 10%, 500 mg to 1 g of benzoic acid may be added, prior to digestion, to facilitate the decomposition of the substance.

**METHOD II (SEMIMICRO)**

Transfer an accurately weighed or measured quantity of the sample, equivalent to about 2 or 3 mg of nitrogen, into the digestion flask of a semimicro Kjeldahl apparatus. Add 1 g of a 10:1 powdered mixture of potassium sulfate and cupric sulfate, using a fine jet of water to wash down any material adhering to the neck of the flask, and then pour 7 mL of sulfuric acid down the inside wall of the flask to rinse it. Cautiously add down the inside of the flask 1 mL of 30% hydrogen peroxide, swirling the flask during the addition.

[CAUTION—Do not add any peroxide during the digestion.]

Heat over a free flame or an electric heater until the solution has attained a clear blue color and the walls of the flask are free from carbonized material. Cautiously add 20 mL of water, then add through a funnel 30 mL of a 2:5 solution of sodium hydroxide, and rinse the funnel with 10 mL of water. Connect the flask to a steam distillation apparatus, and immediately begin the distillation with steam. Collect the distillate in 15 mL of a 1:25 solution of boric acid to which has been added 3 drops of *methyl red*-methylene blue *TS*. When the nitrogen content of the distillate is determined by titration with 0.02 or 0.1 N sulfuric acid, each mL of 0.01 N acid is equivalent to 140 µg of nitrogen.

When more than 2 or 3 mg of nitrogen is present in the measured quantity of the substance to be determined, 0.02 or 0.1 N sulfuric acid may be used in the titration if at least 15 mL of titrant is required. If the total dry weight of the material taken is greater than 100 mg, increase proportionately the quantities of sulfuric acid and sodium hydroxide added before distillation.

**SELENIUM(IV) AND SELENIUM(VI) SPECIATION**

**Principle:** As concentrations slightly above those required for health can induce toxicity, it is highly important that selenium determination be precise and specific. The following method utilizes an HPLC-based separation of the sample hyphenated with ICP-MS for the determination of Se(IV) and Se(VI) in potassium selenate. An anion exchange column is used to separate Se(IV) from Se(VI), and an ICP-MS equipped with a collision-reaction cell technology (CCT) was applied to provide sensitive and specific analysis of different oxidation states of selenium by removing the matrix-based polyatomic interferences.

[NOTE—Use high-purity deionized water (>18 MΩ · cm at 25°) to prepare all solutions. Use solvents that are of sufficient purity for analysis of trace contaminants.]

**Mobile phase (11 mM ammonium citrate buffer, pH 5.0):** In a 1000-mL volumetric flask, dissolve 2.10 g of citric acid in about 900 mL of water, adjust the pH of the resulting solution with ammonium hydroxide solution (28%–30% ammonia) to 5.0, and dilute with water to volume.

**Standard stock solution A:** Use commercially available standard solution containing 1000 mg/L Se(IV).² Dilute an aliquot of this solution with water to obtain *Standard stock solution A* containing 11.58 mg/L Se(IV)².

**Standard stock solution B:** Use commercially available standard solution containing 878 mg/L of Se(VI) and 157 mg/L of Se(IV)⁸. Dilute an aliquot of this solution and water to obtain *Standard stock solution B* containing 8.78 mg/L Se(VI) with 1.57 mg/L Se(IV).

**Standard solutions:** Dilute individual aliquots of *Standard stock solution A* and *Standard stock solution B* with water to obtain *Standard solutions* containing a range of 2–250 µg/L Se(IV).

**Sample solution:** Dilute a portion of sample to a final concentration within the calibration range with water (0.2 mg/mL Se(IV)).

**Chromatographic system, Appendix IIA**

**Mode:** HPLC (coupled to ICP-MS with collision-reaction cell technology)

**Detector:** ICP-MS with collision-reaction cell technology. [Note—The instrument and its operational parameters are described in the Elemental spectrometric system section.]

**Column:** 4.1-mm × 250-mm, 10-µm particle size anion exchange; with microporous substrate, fully functionalized with trimethylamine groups.⁹

**Column temperature:** Ambient

**Injection volume:** 100 µL

**Flow rate:** 1.0 mL/min

**Elemental spectrometric system, Appendix IIIIC**

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² Available from Inorganic Ventures, catalog numbers: CGSE(4)1-1, CGSE(4)1-2, or CGSE(4)1-5, Christiansburg, VA 24073; or equivalent.

⁸ Available from Inorganic Ventures, catalog numbers: CGSE(6)1-1 or CGSE(6)1-5, Christiansburg, VA 24073; or equivalent.

⁹ Hamilton model PRP-X100 Anion Exchange, PN 79433, available from Hamilton Company, 4970 Energy Way, Reno, NV 89502 USA; or equivalent.
Mode: ICP using collision-reaction cell technology (CCT) mode. [NOTE—The spray chamber of the instrument should be cooled to 2° with a Peltier-type cooling device.]

Detector: Mass spectrometer
ICP RF power: 1400 W
Flow rates
- Plasma argon: 13 L/min
- Auxiliary argon: 0.7 L/min
- Nebulizer gas: 0.84 L/min
- CCT gas: 7% hydrogen in helium; 5.35 mL/min

Extraction lens voltage: -161 V
Pole bias: -16 V
Hexapole bias: -20 V

Data mode: Time-resolved analysis

Calibration: Separately introduce each of the Standard solutions to the ICP-MS according to the instructions supplied with the instrument. [NOTE—Chromatography is not to be performed on the Standard solutions.] Use an aliquot of water as a standard solution with a Se(IV) and Se(VI) concentration of 0 µg/L. Record the results and construct a calibration curve with the integrated peak area (of the species intensity) on the y-axis and the concentration of Se(IV) or Se(VI) in the Standard solutions on the x-axis (µg/L).

Analysis: Inject the Sample solution into the chromatograph and record the resulting chromatogram. The approximate retention times for Se(IV) and Se(VI) are 187,000 ms (3.1 min) and 437,000 ms (7.3 min). Record the results of the ICP-MS analysis of Se(IV) and Se(VI), in µg/mL, according to the calibration curve previously generated. Calculate the amount of Se(IV) or Se(VI), as needed, in the sample taken (µg/g):

\[ \text{Result} = \frac{r_U}{C_U} \]

- \( r_U \) = concentration of Se(IV) or Se(VI) in the Sample solution, as determined from comparison of the ICP-MS intensity of the Sample solution to the calibration curve (µg/L)
- \( C_U \) = concentration of the Sample solution (mg/mL)

SPECTROPHOTOMETRIC IDENTIFICATION TESTS

Spectrophotometric tests contribute meaningfully toward the identification of many compendial chemical substances. The test procedures that follow are applicable to substances that absorb IR and/or UV radiation. The IR absorption spectrum of a substance, compared with that obtained concomitantly for the corresponding USP Reference Standard, provides perhaps the most conclusive evidence of the identity of the substance that can be realized from any single test. The UV absorption spectrum, on the other hand, does not exhibit a high degree of specificity. Conformance with both IR absorption and UV absorption test specifications leaves little doubt, if any, regarding the identity of the specimen under examination.

• INFRARED SPECTRA
  This test is used for comparison of an IR spectrum for a sample specimen with a reference spectrum provided in the individual monograph.
  Sample specimens should be prepared using the same technique as that used for the provided spectra. Unless otherwise noted in the individual monograph or spectrum caption, the spectra for liquid substances were obtained on neat liquids contained in fixed-volume sodium chloride cells or between salt plates. Spectra for solid substances were obtained on a potassium bromide pellet or a mineral oil (Nujol or equivalent) dispersion, as indicated in individual monographs or spectrum caption.

• INFRARED ABSORPTION
  This test is used for comparison of the IR spectrum of a sample specimen with that of a physical USP Reference Standard. Sample and USP Reference Standard specimens should both be prepared for analysis using the same technique, as directed in the individual monographs, which use the below letter designations. Sample and USP Reference Standard specimens should be used as either dried or undried specimens as directed on the Reference Standard Label.
  Record the spectra of the sample and USP Reference Standard specimens over the range of about 2.6–15 µm (3800 cm\(^{-1}\) to 650 cm\(^{-1}\)) unless otherwise specified in the individual monograph.

<table>
<thead>
<tr>
<th>Designation</th>
<th>Specimen Preparation Technique</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Intimately in contact with an internal reflection element for attenuated total reflectance (ATR) analysis</td>
</tr>
<tr>
<td>E</td>
<td>Pressed as a thin sample against a suitable plate for IR microscopic analysis</td>
</tr>
<tr>
<td>F</td>
<td>Suspended neat between suitable (for example sodium chloride or potassium bromide) plates</td>
</tr>
<tr>
<td>K</td>
<td>Mixed intimately with potassium bromide and compressed into a translucent pellet</td>
</tr>
<tr>
<td>M</td>
<td>Finely ground and dispersed in mineral oil</td>
</tr>
<tr>
<td>S</td>
<td>A solution of designated concentration, prepared as directed in the individual monograph, and examined in 0.1-mm cells (unless otherwise specified in the individual monograph)</td>
</tr>
</tbody>
</table>

[NOTE—A and E techniques can be used as alternative methods for K, M, F, and S where testing is performed qualitatively and the Reference Standard spectra are similarly obtained.]
Differences that may be observed in the IR spectra obtained for the analyte and the standard sometimes are attributed to the presence of polymorphs, which are not always acceptable. Polymorphism gives rise to variations in the IR spectra of many compounds in the solid state. Frequently, small differences in structure result in significant differences in the spectra. Unless otherwise directed in the individual monograph, therefore, continue as follows. If a difference appears in the IR spectra of the analyte and the standard, dissolve equal portions of the test specimen and the Reference Standard in equal volumes of a suitable solvent, evaporate the solution to dryness in similar containers under identical conditions, and repeat the test on the residues.

**ULTRAVIOLET ABSORPTION**

The test is used for comparison of the UV spectrum of a sample specimen with that of a physical USP Reference Standard. Sample and USP Reference Standard specimens should be prepared using the solvents and concentrations specified in the individual monograph. Record the spectra of the sample and USP Reference Standard solutions concomitantly using 1-cm cells over the spectral range of 200–400 nm, unless otherwise indicated in the individual monograph. Calculate absorptivities and/or absorbance ratios where these criteria are included in an individual monograph. Unless otherwise specified, absorbances indicated for these calculations are those measured at the maximum absorbance at the wavelength specified in the individual monograph. Where the absorbance is to be measured at a wavelength other than that of maximum absorbance, the abbreviations (min) and (sh) are used to indicate a minimum and shoulder, respectively, in an absorbance spectrum.

**SULFUR** (by Oxidative Microcoulometry) (Based on ASTM D3120)\(^\text{10}\)

[Note—All reagents used in this test should be reagent grade; water should be of high purity, and gases must be high-purity grade.]

**Apparatus:** Use the Dohrmann Microcoulometric Titrating System (MCTS-30), or equivalent (shown in Figure 20), unless otherwise specified in an individual monograph. It consists of a constant rate injector, A, a pyrolysis furnace, B, a quartz pyrolysis tube, C, a granular-tin scrubber, D, a titration cell, E, and a microcoulometer with a digital readout, F.

**Figure 20. Microcoulometric Titrating System for the Determination of Sulfur in Hexanes**

**Granular-tin scrubber:** Place 5 g of 20- to 30-mesh granular reagent-grade tin between quartz-wool plugs in an elongated 18/9-12/5 standard-taper adaptor that connects the pyrolysis tube and the titration cell.

**Microcoulometer:** Must have variable attenuation; gain control; and be capable of measuring the potential of the sensing-reference electrode pair, and comparing this potential with a bias potential, amplifying the potential difference, and applying the amplified difference to the working-auxiliary electrode pair to generate a titrant. The microcoulometer output voltage signal must also be proportional to the generating current.

**Pyrolysis furnace:** The sample should be pyrolyzed in an electric furnace having at least two separate and independently controlled temperature zones, the first being an inlet section that can maintain a temperature sufficient to volatilize all the organic sample. The second zone is a pyrolysis section that can maintain a temperature sufficient to pyrolyze the organic matrix and oxidize all the organically bound sulfur. A third outlet temperature zone is optional.

**Pyrolysis tube:** Must be fabricated from quartz and constructed in such a way that a sample, which is vaporized completely in the inlet section, is swept into the pyrolysis zone by an inert gas where it mixes with oxygen and is burned. The inlet end of the tube shall hold a septum for syringe entry of the sample and side arms for the introduction of oxygen and inert gases. The center or pyrolysis section should be of sufficient volume to ensure complete pyrolysis of the sample.

**Sampling syringe:** A microlitre syringe of 10-µL capacity capable of accurately delivering 1–10 µL of sample into the pyrolysis tube. Three-in × 24-gauge needles are recommended to reach the inlet zone of the pyrolysis furnace.

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\(^{10}\) Adapted from ASTM D3120 Standard Test Method for Trace Quantities of Sulfur in Light Liquid Petroleum Hydrocarbons by Oxidative Microcoulometry. The original ASTM method is available in its entirety from ASTM International, 100 Barr Harbor Drive, West Conshohocken, PA 19428. Phone: 610-832-9385, Fax: 610-832-9555, Email: service@astm.org, Website: www.astm.org.
Titration cell: Must contain a sensor-reference pair of electrodes to detect changes in triiodide ion concentration and a generator anode-cathode pair of electrodes to maintain constant triiodide ion concentration and an inlet for a gaseous sample from the pyrolysis tube. The sensor electrode shall be platinum foil and the reference electrode platinum wire in saturated triiodide half-cell. The generator anode and cathode half-cell shall also be platinum. The titration cell shall be placed on a suitable magnetic stirrer.

Preparation of apparatus: Carefully insert the quartz pyrolysis tube into the furnace, attach the tin scrubber, and connect the reactant and carrier-gas lines. Add the Cell electrolyte solution (see below) to the titration cell, and flush the cell several times. Maintain an electrolyte level of 3.2–6.6 mm above the platinum electrodes. Place the titration cell on a magnetic stirrer, and connect the cell inlet to the tin scrubber outlet. Position the platinum-foil electrodes (mounted on the movable cell head) so that the gas-inlet flow is parallel to the electrodes with the generator anode adjacent to the generator cathode. Assemble and connect the coulometer in accordance with the manufacturer’s instructions. Double-wrap the adaptor containing the tin scrubber with heating tape and turn the heating tape on. Adjust the flow of the gases, the pyrolysis furnace temperature, the titration cell, and the coulometer to the desired operating conditions. Typical operating conditions are as follows:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reactant gas flow (oxygen), cm³/min</td>
<td>200</td>
</tr>
<tr>
<td>Carrier-gas flow (Ar, He), cm³/min</td>
<td>40</td>
</tr>
<tr>
<td>Furnace temperature, °C Inlet zone</td>
<td>700 (maximum)</td>
</tr>
<tr>
<td>Pyrolysis zone</td>
<td>800–1000</td>
</tr>
<tr>
<td>Outlet zone</td>
<td>800 (maximum)</td>
</tr>
<tr>
<td>Tin-scrubber temperature, °C</td>
<td>200</td>
</tr>
<tr>
<td>Titrator cell</td>
<td>Stirrer speed set to produce slight vortex</td>
</tr>
<tr>
<td>Coulometer</td>
<td></td>
</tr>
<tr>
<td>Bias voltage, mV</td>
<td>160</td>
</tr>
<tr>
<td>Gain</td>
<td>50</td>
</tr>
<tr>
<td>Constant Rate Injector, µL/s</td>
<td>0.25</td>
</tr>
</tbody>
</table>

The tin scrubber must be conditioned to sulfur, nitrogen, and chlorine before quantitative analysis can be achieved. A solution containing 10 mg/kg of butyl sulfide, 100 mg/kg of pyridine, and 200 mg/kg of chlorobenzene in isoctane has proven an effective conditioning agent. With a fresh scrubber installed and heated, two 30-µL samples of this conditioning agent injected at a flow rate of 0.5 µL/s produce a steadily increasing response, with final conditioning indicated by a constant reading from the offset during the second injection.

Reagents
Argon or helium (argon preferred): High-purity grade, used as the carrier gas. Two-stage gas regulators must be used.
Cell electrolyte solution: Dissolve 0.5 g of potassium iodide and 0.6 g of sodium azide in 500 mL of high-purity water, add 5 mL of glacial acetic acid, and dilute to 1 L. Store in a dark bottle or in a dark place and prepare fresh at least every 3 months.
Oxygen: High-purity grade, used as the reactant gas
Iodine: Resublimed, 20-mesh or less, for saturated reference electrode
Sulfur standard (approximately 100 mg/kg): Transfer 0.1569 g of n-butyl sulfide, accurately weighed, into a tared 500-mL volumetric flask. Dilute to the mark with isoctane, and reweigh. Calculate the sulfur concentration (S), as a percentage:

\[ S = \frac{W_b}{W_i} \times 2.192 \times 10^5 \]

in which \( W_b \) is the weight of n-butyl sulfide and \( W_i \) is the weight of the solution.

Calibration: Prepare a calibration standard (approximately 5 mg/kg) by pipetting 5 mL of Sulfur standard into a 10-mL volumetric flask and diluting with isoctane to volume. Fill and clamp the syringe onto the constant-rate injector, push the sliding carriage forward to penetrate the septum with the needle, and zero the meter in case of long-term drift in the automatic baseline zero circuitry. Switch \( S_1 \) automatically starts the stepper-motor syringe drive and initiates the analysis cycle. At 2.5 min (before the 3-min meter hold point) set the digital meter with the scan potentiometer to correspond to the sulfur content of the known standard to the nearest 0.01 mg/kg. At the 3-min point, the number displayed on the meter stops, the plunger drive block is retracted to its original position, as preset by switch \( S_2 \), and a baseline re-equilibration period equal to the injection period elapses before a ready light and a beeper indicate that a new sample may be injected. Repeat the Calibration step a total of at least four times.

Procedure: Rinse the syringe several times with sample; then fill it, clamp it onto the constant-rate injector, push the sliding carriage forward to penetrate the septum with the needle, and zero the meter. Turn on switch \( S_1 \) to start the stepper-motor syringe drive automatically and initiate the analysis cycle. After the 3-min hold point, the number displayed on the meter corresponds to the sulfur content of the injected sample.
Figure 21. Raney Nickel Reduction Apparatus
APPENDIX XIII: ADULTERANTS AND CONTAMINANTS IN FOOD INGREDIENTS

Tests contained within this Appendix are not monograph requirements, but rather are provided as informational methods for FCC users.

DIETHYLENE GLYCOL AND ETHYLENE GLYCOL IN GLYCERIN

This method was developed for the identification and quantification of low levels of ethylene glycol and diethylene glycol in glycerin.

**Standard solution:** 0.025 mg/mL of USP Ethylene Glycol RS, 0.025 mg/mL of USP Diethylene Glycol RS, 50 mg/mL of USP Glycerin RS, and 0.05 mg/mL of 2,2,2-trichloroethanol (internal standard) in methanol

**Sample solution:** 50 mg/mL of sample and 0.05 mg/mL of 2,2,2-trichloroethanol (internal standard) in methanol

**Chromatographic system, Appendix IIA**

**Mode:** GC

**Detector:** Flame ionization

**Column:** 0.53-mm × 30-m fused-silica analytical; coated with 3.0-µm 6% cyanopropylphenyl–94% dimethylpolysiloxane stationary phase

**Temperatures**

**Injector:** 220°

**Detector:** 250°

**Column:** See the temperature program table below.

<table>
<thead>
<tr>
<th>Initial Temperature (°)</th>
<th>Temperature Ramp (°/min)</th>
<th>Final Temperature (°)</th>
<th>Hold Time at Final Temperature (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>—</td>
<td>100</td>
<td>4</td>
</tr>
<tr>
<td>100</td>
<td>50</td>
<td>120</td>
<td>10</td>
</tr>
<tr>
<td>120</td>
<td>50</td>
<td>220</td>
<td>6</td>
</tr>
</tbody>
</table>

**Carrier gas:** Helium

**Injection volume:** 1.0 µL

**Flow rate:** 4.5 mL/min

**Injection type:** Split flow ratio is about 10:1.

**System suitability**

**Sample:** Standard solution

**Suitability requirements**

**Resolution:** NLT 1.5 between diethylene glycol and glycerin

**Analysis:** Separately inject equal volumes of the Standard solution and Sample solution into the chromatograph, and measure the responses for the major peaks on the resulting chromatograms. Diethylene glycol and ethylene glycol can be identified in the Sample solution on the basis of peak retention times compared to those in the Standard solution.

[NOTE—The relative retention times for ethylene glycol, 2,2,2-trichloroethanol, diethylene glycol, and glycerin are about 0.3, 0.6, 0.8, and 1.0, respectively. See Figure 1 for example chromatograms.] The percentages of diethylene glycol and ethylene glycol in the portion of sample taken are calculated using the following formula:

\[
\text{Result} = \left(\frac{R_U}{R_S}\right) \times \left(\frac{C_S}{C_U}\right) \times 100
\]

\(R_U = \) analyte relative response (analyte peak response/2,2,2-trichloroethanol peak response) from the Sample solution

\(R_S = \) analyte relative response (analyte peak response/2,2,2-trichloroethanol peak response) from the Standard solution

\(C_S = \) concentration of analyte in the Standard solution (mg/mL)

\(C_U = \) concentration of sample in the Sample solution (mg/mL)

**Performance characteristics**

**Limit of quantitation:** 0.025% (w/w) for ethylene glycol and diethylene glycol

**Range:** 0.013–0.031 mg/mL for ethylene glycol and 0.012–0.030 mg/mL for diethylene glycol. [NOTE—Wider linear ranges may be achievable but were not investigated when developing this method.]

---


2 DB-624 (J & W Scientific), or equivalent.
PESTICIDE RESIDUES

The methods and information contained in this section are designed to measure pesticide residues in food ingredients of botanical origin.

• GENERAL METHOD FOR PESTICIDE RESIDUES ANALYSIS

Definition
Where used in this compendium, the designation pesticide applies to any substance or mixture of substances intended to prevent, destroy, or control any unwanted species of plants or animals causing harm during or otherwise interfering with the production, processing, storage, transport, or marketing of pure articles. The designation includes substances intended for use as growth regulators, defoliants, or desiccants, and any substance applied to crops before or after harvest to protect the product from deterioration during storage and transport.

Limits
Limits for pesticides for foods are determined by the Environmental Protection Agency (EPA), and where no limit is set, the limit is zero. The limits contained in Table 1, therefore, may not be applicable in the United States and are provided for guidance purposes only, and not for the purpose of meeting a regulatory requirement in the United States. The limits may be applicable in other countries where the presence of pesticide residues is permitted. Unless otherwise specified in the individual monograph, the article under test contains NMT the amount of any pesticide indicated in Table 1.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Limit (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alachlor</td>
<td>0.02</td>
</tr>
<tr>
<td>Aldrin and Dieldrin (sum of)</td>
<td>0.05</td>
</tr>
<tr>
<td>Azinphos-methyl</td>
<td>1.0</td>
</tr>
<tr>
<td>Bromopropylate</td>
<td>3.0</td>
</tr>
<tr>
<td>Chlordane (sum of cis- and trans- isomers and oxychlordane)</td>
<td>0.05</td>
</tr>
<tr>
<td>Chlorfenvinphos</td>
<td>0.5</td>
</tr>
<tr>
<td>Chlorpyrifos</td>
<td>0.2</td>
</tr>
<tr>
<td>Chlorpyrifos-methyl</td>
<td>0.1</td>
</tr>
</tbody>
</table>
### Table 1 (continued)

<table>
<thead>
<tr>
<th>Substance</th>
<th>Limit (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cypermethrin (and isomers)</td>
<td>1.0</td>
</tr>
<tr>
<td>DDT (sum of p,p'-DDT, o,p'-DDT, p,p'-DDE, and p,p'-TDE)</td>
<td>1.0</td>
</tr>
<tr>
<td>Deltamethrin</td>
<td>0.5</td>
</tr>
<tr>
<td>Diazinon</td>
<td>0.5</td>
</tr>
<tr>
<td>Dichlorvos</td>
<td>1.0</td>
</tr>
<tr>
<td>Dithiocarbamates (as CS&lt;sub&gt;2&lt;/sub&gt;)</td>
<td>2.0</td>
</tr>
<tr>
<td>Endosulfan (sum of endosulfan isomers and endosulfan sulfate)</td>
<td>3.0</td>
</tr>
<tr>
<td>Endrin</td>
<td>0.05</td>
</tr>
<tr>
<td>Ethion</td>
<td>2.0</td>
</tr>
<tr>
<td>Fenitrothion</td>
<td>0.5</td>
</tr>
<tr>
<td>Fenvalerate</td>
<td>1.5</td>
</tr>
<tr>
<td>Fonofos</td>
<td>0.05</td>
</tr>
<tr>
<td>Heptachlor (sum of heptachlor and heptachlor epoxide)</td>
<td>0.05</td>
</tr>
<tr>
<td>Hexachlorobenzene</td>
<td>0.1</td>
</tr>
<tr>
<td>Hexachlorocyclohexane isomers (other than γ)</td>
<td>0.3</td>
</tr>
<tr>
<td>Lindane (γ-hexachlorocyclohexane)</td>
<td>0.6</td>
</tr>
<tr>
<td>Malathion</td>
<td>1.0</td>
</tr>
<tr>
<td>Methidathion</td>
<td>0.2</td>
</tr>
<tr>
<td>Parathion</td>
<td>0.5</td>
</tr>
<tr>
<td>Parathion-methyl</td>
<td>0.2</td>
</tr>
<tr>
<td>Permethrin</td>
<td>1.0</td>
</tr>
<tr>
<td>Phosalone</td>
<td>0.1</td>
</tr>
<tr>
<td>Piperonyl butoxide</td>
<td>3.0</td>
</tr>
<tr>
<td>Pirimiphos-methyl</td>
<td>4.0</td>
</tr>
<tr>
<td>Pyrethrins (sum of)</td>
<td>3.0</td>
</tr>
<tr>
<td>Quintozene (sum of quintozene, pentachloroaniline and methyl pentachlorophenyl sulfide)</td>
<td>1.0</td>
</tr>
</tbody>
</table>

### Sampling

For articles in containers holding less than 1 kg, mix the contents, and withdraw a quantity sufficient for the tests. For articles in containers holding between 1 and 5 kg, withdraw equal portions from the upper, middle, and lower parts of the container, each of the samples being sufficient to carry out the tests. Thoroughly mix the samples, and withdraw an amount sufficient to carry out the tests. For containers holding more than 5 kg, withdraw three samples, each weighing NLT 250 g, from the upper, middle, and lower parts of the container. Thoroughly mix the samples, and withdraw a portion sufficient to carry out the tests.

If the number of the containers, n, is three or fewer, withdraw samples from each container as indicated above. If the number of containers is more than three, take samples from √n + 1 containers, rounding up to the nearest whole number if necessary.

[Note—Conduct tests without delay to avoid possible degradation of the residues. If this is not possible, store the samples in hermetic containers suitable for food contact, at a temperature below 0, and protected from light.]

### Reagents

Use reagents and solvents that are free from any contaminants, especially pesticides, that might interfere with the analysis. It is often necessary to use special grade solvents suitable for pesticide residue analysis or solvents that have recently been redistilled in an apparatus made entirely of glass. In any case, suitable blank tests must be performed.

### Preparation of Apparatus

Clean all equipment, especially glassware, to ensure that it is free from pesticides. Soak all glassware for a minimum of 16 h in a solution of phosphate-free detergent, rinse with copious quantities of distilled water, and then wash with acetone, followed by hexane or heptane.

### Qualitative and Quantitative Analysis of Pesticide Residues

Use validated analytical procedures that satisfy the following criteria. The method, especially with respect to its purification steps, is suitable for the combination of pesticide residue and substance under test, and is not susceptible to interference from co-extractives. Measure the limits of detection and quantification for each pesticide matrix combination to be analyzed; the method is shown to recover between 70% and 110% of each pesticide; the repeatability and reproducibility of the method are NLT the appropriate values indicated in Table 2; and the...
concentrations of test and reference solutions and the setting of the apparatus are such that a linear response is obtained from the analytical detector.

<table>
<thead>
<tr>
<th>Table 2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Concentration of Pesticide</strong> (mg/kg)</td>
</tr>
<tr>
<td>0.010</td>
</tr>
<tr>
<td>0.100</td>
</tr>
<tr>
<td>1.000</td>
</tr>
</tbody>
</table>

**Test for Pesticides**

Depending on the substance being examined, it may be necessary to modify, sometimes extensively, the procedure described hereafter. Additionally, it may be necessary to perform another method with another column having a different polarity, another detection method (e.g., mass spectrometry), or a different method (e.g., immunochemical method) to confirm the results.

**Extraction:** [Note—Use the following procedure for the analysis of samples and articles having a water content of less than 15%. Samples having a higher water content may be dried, provided that the drying procedure does not significantly affect the pesticide content.]

To 10 g of the coarsely powdered substance under test, add 100 mL of acetone, and allow to stand for 20 min. Add 1 mL of a solution in toluene containing 1.8 μg/mL of carbofenthion. Mix in a high-speed blender for 3 min. Filter this solution, and wash the residue with two 25-mL portions of acetone. Combine the filtrate and the washings, and heat, in a rotary evaporator, maintaining the temperature of the bath below 40° until the solvent has almost completely evaporated. To the residue add a few mL of toluene, and heat again until the acetone is completely removed. Dissolve the residue in 8 mL of toluene. Pass through a membrane filter having a 45-μm porosity, rinse the flask and the filter with toluene, and dilute with toluene to 10.0 mL. This is Solution A.

**Purification**

**Organochlorine, organophosphorus, and pyrethroid insecticides:** The size-exclusion chromatograph is equipped with a 7.8-mm × 30-cm stainless steel column containing 5-μm packing of styrene-divinylbenzene copolymer. Toluene is used as the mobile phase at a flow rate of 1 mL/min.

**Performance of the column:** Inject 100 μL of a solution in toluene containing, in each mL, 0.5 mg of methyl red and 0.5 mg of oracet blue. The column is not suitable unless the color of the eluate changes from orange to blue at an elution volume of about 10.3 mL. If necessary, calibrate the column, using a solution in toluene containing suitable concentrations of the pesticide of interest having the lowest molecular weight (for example, dichlorvos) and that having the highest molecular weight (for example, deltamethrin). Determine which fraction of the eluate contains both pesticides.

**Purification of the sample solution:** Inject a suitable volume (100–500 μL) of Solution A into the chromatograph. Collect the fraction (Solution B) as determined above under Performance of the column. Organophosphorus pesticides elute between 8.8 and 10.9 mL. Organochlorine and pyrethroid pesticides elute between 8.5 and 10.3 mL.

**Organochlorine and pyrethroid insecticides:** Into a 5-mm × 10-cm chromatographic column, introduce a piece of fat-free cotton and 0.5 g of silica gel treated as follows. Heat chromatographic silica gel in an oven at 150° for NLT 4 h. Allow to cool, and add dropwise a quantity of water corresponding to 1.5% of the weight of the silica gel used. Shake vigorously until agglomerates have disappeared, and continue shaking by mechanical means for 2 h. Condition the column with 1.5 mL of hexane. [Note—Prepacked columns containing about 0.50 g of a suitable silica gel may also be used, provided they have been previously validated.]

Concentrate Solution B almost to dryness, with the aid of a stream of helium or oxygen-free nitrogen, and dilute with toluene to a suitable volume (200 μL to 1 mL, according to the volume injected in the preparation of Solution B). Quantitatively transfer this solution to the column, and proceed with the chromatography, using 1.8 mL of toluene as the mobile phase. Collect the eluate (Solution C).

**Quantitative Analysis of Organophosphorus Insecticides**

**Sample solution:** Concentrate Solution B almost to dryness, with the aid of a stream of helium, and dilute with toluene to 100 μL.

**Standard solution:** Prepare at least three solutions in toluene containing each of the pesticides of interest and carbofenthion at concentrations suitable for plotting a calibration curve.

**Chromatographic system, Appendix II A**

**Mode:** GC

**Detector:** Flame-ionization (alkali) or flame-photometric

**Column:** 0.32-mm × 30-m fused silica; coated with a 0.25-μm layer of dimethylpolysiloxane

**Carrier gas:** Hydrogen (may also use helium or nitrogen)

**Temperatures**

**Injector:** 250°

**Detector:** 275°

**Column:** See the temperature program table below.
Initial Temperature (°) | Temperature Ramp (°/min) | Final Temperature (°) | Hold Time at Final Temperature (min)
---|---|---|---
80 | — | 80 | 1
80 | 30 | 150 | 3
150 | 4 | 280 | 1

**Analysis:** Use carbophenothion as the internal standard. [Note—If necessary, use a second internal standard to identify any possible interference with the peak corresponding to carbophenothion.] Inject the chosen volume of each solution, record the chromatograms, and measure the peak responses. [Note—The approximate relative retention times are listed in Table 3.] Calculate the content of each pesticide from the peak areas and the concentrations of the solution.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Relative Retention Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dichlorvos</td>
<td>0.20</td>
</tr>
<tr>
<td>Fonofos</td>
<td>0.50</td>
</tr>
<tr>
<td>Diazinon</td>
<td>0.52</td>
</tr>
<tr>
<td>Parathion-methyl</td>
<td>0.59</td>
</tr>
<tr>
<td>Chlorpyrifos-methyl</td>
<td>0.60</td>
</tr>
<tr>
<td>Pirimiphos-methyl</td>
<td>0.66</td>
</tr>
<tr>
<td>Malathion</td>
<td>0.67</td>
</tr>
<tr>
<td>Parathion</td>
<td>0.69</td>
</tr>
<tr>
<td>Chlorpyrifos</td>
<td>0.70</td>
</tr>
<tr>
<td>Methidathion</td>
<td>0.78</td>
</tr>
<tr>
<td>Ethion</td>
<td>0.96</td>
</tr>
<tr>
<td>Carbophenothion</td>
<td>1.00</td>
</tr>
<tr>
<td>Azinphos-methyl</td>
<td>1.17</td>
</tr>
<tr>
<td>Phosalone</td>
<td>1.18</td>
</tr>
</tbody>
</table>

**Quantitative Analysis of Organochlorine and Pyrethroid Insecticides**

**Sample solution:** Concentrate Solution C almost to dryness, with the aid of a stream of helium or oxygen-free nitrogen, and dilute with toluene to 500 µL.

**Standard solution:** Prepare at least three solutions in toluene containing each of the pesticides of interest and carbophenothion at concentrations suitable for plotting a calibration curve.

**Chromatographic system, Appendix IIA**

**Mode:** GC

**Detector:** Electron capture

**Column:** 0.32-mm × 30-m fused silica; coated with a 0.25-µm layer of dimethylpolysiloxane

**Carrier gas:** Hydrogen (may also use helium or nitrogen)

**Temperatures**

**Injector:** 275°

**Detector:** 300°

**Column:** See the temperature program table below.

| Initial Temperature (°) | Temperature Ramp (°/min) | Final Temperature (°) | Hold Time at Final Temperature (min)
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>80</td>
<td>—</td>
<td>80</td>
<td>1</td>
</tr>
<tr>
<td>80</td>
<td>30</td>
<td>150</td>
<td>3</td>
</tr>
<tr>
<td>150</td>
<td>4</td>
<td>280</td>
<td>1</td>
</tr>
</tbody>
</table>

**Analysis:** Use carbophenothion as the internal standard. [Note—If necessary, use a second internal standard to identify any possible interference with the peak corresponding to carbophenothion.] Inject the chosen volume of each solution, record the chromatograms, and measure the peak responses. [Note—The approximate relative retention times are listed in Table 4.] Calculate the content of each pesticide from the peak areas and the concentrations of the solution.

Published on March 26, 2020
### Table 4

<table>
<thead>
<tr>
<th>Substance</th>
<th>Relative Retention Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Hexachlorocyclohexane</td>
<td>0.44</td>
</tr>
<tr>
<td>Hexachlorobenzene</td>
<td>0.45</td>
</tr>
<tr>
<td>β-Hexachlorocyclohexane</td>
<td>0.49</td>
</tr>
<tr>
<td>Lindane</td>
<td>0.49</td>
</tr>
<tr>
<td>δ-Hexachlorocyclohexane</td>
<td>0.54</td>
</tr>
<tr>
<td>ε-Hexachlorocyclohexane</td>
<td>0.56</td>
</tr>
<tr>
<td>Heptachlor</td>
<td>0.61</td>
</tr>
<tr>
<td>Aldrin</td>
<td>0.68</td>
</tr>
<tr>
<td>cis-Heptachlor epoxide</td>
<td>0.76</td>
</tr>
<tr>
<td>o,p'-DDE</td>
<td>0.81</td>
</tr>
<tr>
<td>α-Endosulfan</td>
<td>0.82</td>
</tr>
<tr>
<td>Dieldrin</td>
<td>0.87</td>
</tr>
<tr>
<td>p,p'-DDE</td>
<td>0.87</td>
</tr>
<tr>
<td>o,p'-DDD</td>
<td>0.89</td>
</tr>
<tr>
<td>Endrin</td>
<td>0.91</td>
</tr>
<tr>
<td>β-Endosulfan</td>
<td>0.92</td>
</tr>
<tr>
<td>o,p'-DDT</td>
<td>0.95</td>
</tr>
<tr>
<td>Carbophenothion</td>
<td>1.00</td>
</tr>
<tr>
<td>p,p'-DDT</td>
<td>1.02</td>
</tr>
<tr>
<td>cis-Permethrin</td>
<td>1.29</td>
</tr>
<tr>
<td>trans-Permethrin</td>
<td>1.31</td>
</tr>
<tr>
<td>Cypermethrin&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.40</td>
</tr>
<tr>
<td>Fenvalerate&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.47</td>
</tr>
<tr>
<td>Deltamethrin</td>
<td>1.49</td>
</tr>
<tr>
<td></td>
<td>1.54</td>
</tr>
</tbody>
</table>

<sup>a</sup> The substance shows several peaks.

---

**Add the following:**

**ADDED COLORS IN SPICES**

The methods and information contained in this section are designed to detect the presence of potentially undeclared colors in spices and spice mixes.

- **Non-Targeted Screening for Synthetic Colors in Spices Using Thin-Layer Chromatography (TLC)**
  - **Principle:** Thin-layer chromatography and paper chromatography are two commonly used methods to detect colors in foods. To protect food integrity and public health, a non-targeted screening method for colors added to spices or spice blends using thin-layer chromatography is introduced. This method examines the chromatogram of authentic spices and determines whether there are abnormal color peaks in the samples present under visual light. For separation and identification of different colors, three different procedures were developed and validated. Users should test a sufficient number of samples to establish chromatograms of authentic spices or spice blends. Users may choose to run one or more of the following procedures depending on the specific colors suspected as adulterants. If no information is available on specific added colors, users may wish to perform all three procedures. Chromatograms of spices or spice blends that do not match the established sample results may warrant further examination using more specific techniques. The spots on the chromatogram can be compared to the spots from the colors listed in Table 5 to identify colors. The detection limit for the colors listed is 1000 ppm. The detection limit can be extended to lower levels with supportive experimental data.
  - **Scope:** This method is applicable to synthetic colors in paprika, chili powder, turmeric, curry, and sumac.
  - **Sample solution:** Transfer 2 g of the powdered spice sample into a 50-mL amber conical centrifuge tube, add 10 mL of water and 10 mL acetonitrile, then add 4 g of magnesium sulfate, 1 g of sodium chloride, and 1.5 g of sodium citrate dihydrate. Shake the tube at 400 rpm for 15 min, centrifuge at 1789 × g for 10 min, and collect the supernatant as the Sample solution.
  - **Diluent:** Acetonitrile and water (8:2, v/v)
**Color stock solutions:** Prepare individual solutions of each of the following colors. Transfer 2 mg of the color into a 20-
ml volumetric flask and add 5 mL of water to dissolve with mixing and sonication. Dilute with acetonitrile to volume.
For Sudan III, Sudan IV, and Para red, use acetone in place of water and acetonitrile to dissolve and dilute the colors.

### Table 5. Colors

<table>
<thead>
<tr>
<th>Colors</th>
<th>CAS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid black 1</td>
<td>1064-48-8</td>
</tr>
<tr>
<td>Orange II (Acid orange 7)</td>
<td>633-96-5</td>
</tr>
<tr>
<td>Ponceau 4R</td>
<td>2611-82-7</td>
</tr>
<tr>
<td>Yellow 5 (Tartrazine)</td>
<td>1934-21-0</td>
</tr>
<tr>
<td>Allura red AC (Food red 17)</td>
<td>25956-17-6</td>
</tr>
<tr>
<td>Carmoisine (Azorubine)</td>
<td>3567-69-9</td>
</tr>
<tr>
<td>Brilliant black BN</td>
<td>2519-30-4</td>
</tr>
<tr>
<td>Yellow 6 (Food yellow 3)</td>
<td>2783-94-0</td>
</tr>
<tr>
<td>Metanil yellow (Acid yellow 36)</td>
<td>587-98-4</td>
</tr>
<tr>
<td>Sudan I</td>
<td>842-07-9</td>
</tr>
<tr>
<td>Sudan II</td>
<td>3118-97-6</td>
</tr>
<tr>
<td>Sudan III</td>
<td>85-86-9</td>
</tr>
<tr>
<td>Sudan IV</td>
<td>85-83-6</td>
</tr>
<tr>
<td>Para red</td>
<td>6410-10-2</td>
</tr>
<tr>
<td>Rhodamine B</td>
<td>81-88-9</td>
</tr>
<tr>
<td>Auramine O</td>
<td>2465-27-2</td>
</tr>
<tr>
<td>Amaranth (Acid red 27)</td>
<td>915-67-3</td>
</tr>
</tbody>
</table>

**Procedure 1**

Thin-Layer Chromatography, Appendix IIA

*Adsorbent:* 0.2-mm layer of reverse-phase modified silica

*Mixed color solution A:* Transfer 1.0 mL of each of the **Color stock solutions** for yellow 5, ponceau 4R, carmoisine, and para red into a 10-mL volumetric flask, and dilute with *Diluent* to volume.

*Mixed color solution B:* Transfer 1.0 mL of each of the above **Color stock solutions** for brilliant black BN, rhodamine B, and allura red AC into a 10-mL volumetric flask and dilute with *Diluent* to volume.

*Application volume:* 10 µL

*Developing solvent system:* 1-Butanol, methyl ethyl ketone, ammonia, and water (125:75:25:50, v/v/v/v)

*Analysis:* Following development, remove the plate from the chamber, dry thoroughly in air, and visualize under white light. [*NOTE—See Table 6 for the approximate Rf values of the colors.*]

### Table 6

<table>
<thead>
<tr>
<th>Color</th>
<th>Rf Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yellow 5 (Tartrazine)</td>
<td>0.42</td>
</tr>
<tr>
<td>Brilliant black BN</td>
<td>0.44</td>
</tr>
<tr>
<td>Ponceau 4R</td>
<td>0.55</td>
</tr>
<tr>
<td>Carmoisine (Azorubine)</td>
<td>0.64</td>
</tr>
<tr>
<td>Rhodamine B</td>
<td>0.66</td>
</tr>
<tr>
<td>Para red</td>
<td>0.72</td>
</tr>
<tr>
<td>Allura red AC (Food red 17)</td>
<td>0.76</td>
</tr>
</tbody>
</table>

**Acceptance criteria:** The spots from *Mixed color solution A* and *Mixed color solution B* are absent from the chromatogram of the **Sample solution**.

**Procedure 2**

Thin-Layer Chromatography, Appendix IIA

*Adsorbent:* 0.2-mm layer of silica

*Mixed color solution C:* Transfer 1.0 mL of each of the **Color stock solutions** for Sudan I, Sudan II, Sudan III, and Sudan IV into a 10-mL volumetric flask and dilute with *Diluent* to volume.

---

3 Merck TLC Silica gel 60 RP-18 F<sub>254</sub>, available at Millipore Sigma (www.emdmillipore.com), catalog #115685, or equivalent (FCC 12)

4 Merck TLC Silica gel 60 F<sub>254</sub>, available at Millipore Sigma (www.emdmillipore.com), catalog #105554, or equivalent (FCC 12)
Application volume: 10 µL
Developing solvent system: n-Hexane and ethyl acetate (85:15, v/v)
Analysis: Following development, remove the plate from the chamber, dry thoroughly in air, and visualize under white light. [Note—See Table 7 for the approximate R_f values of the colors.]

<table>
<thead>
<tr>
<th>Color</th>
<th>R_f Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sudan III</td>
<td>0.27</td>
</tr>
<tr>
<td>Sudan IV</td>
<td>0.31</td>
</tr>
<tr>
<td>Sudan I</td>
<td>0.35</td>
</tr>
<tr>
<td>Sudan II</td>
<td>0.39</td>
</tr>
</tbody>
</table>

Acceptance criteria: The spots from the Mixed color solution C are absent from the chromatogram of the Sample solution.

Procedure 3
Thin-Layer Chromatography, Appendix IIA
Adsorbent: 0.2-mm layer of silica
Mixed color solution D: Transfer 1.0 mL of each of the Color stock solution for amaranth, yellow 6, acid black, orange II, metanil yellow, and auramine O into a 10-mL volumetric flask and dilute with Diluent to volume.
Application volume: 10 µL
Developing solvent system: 1-Butanol, water, and formic acid (40:15:10, v/v/v)
Analysis: Following development, remove the plate from the chamber, dry thoroughly in air, and visualize under white light. [Note—See Table 8 for the approximate R_f values of the colors.]

<table>
<thead>
<tr>
<th>Color</th>
<th>R_f Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amaranth (Acid red 27)</td>
<td>0.08</td>
</tr>
<tr>
<td>Yellow 6 (Food yellow 3)</td>
<td>0.24</td>
</tr>
<tr>
<td>Acid black 1</td>
<td>0.38</td>
</tr>
<tr>
<td>Orange II (Acid orange 7)</td>
<td>0.52</td>
</tr>
<tr>
<td>Metanil yellow (Acid yellow 36)</td>
<td>0.58</td>
</tr>
<tr>
<td>Auramine O</td>
<td>0.67</td>
</tr>
</tbody>
</table>

Acceptance criteria: The spots from Mixed color solution D are absent from the chromatogram of the Sample solution.▲ (FCC 12)