Parenteral Quality Control

Sterility, Pyrogen, Particulate, and Package Integrity Testing

Third Edition, Revised and Expanded

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To my mother
—D.L.

Preface

Drug products administered by injection are characterized by three qualities possessed by no other type of pharmaceutical dosage form: sterility, freedom from pyrogenicity, and freedom from particulate matter. The achievement of sterile, nonpyrogenic, and particulate-free parenteral products provides a significant challenge to the ingenuity and creativity of parenteral scientists and technologists. Of equal challenge are the successful application and performance of analytical testing procedures to verify the claims of parenteral products that they are indeed, sterile, pyrogen-free, and free from visible particulate contamination.

Official compendial tests for sterility, pyrogens, and particulates evoke widespread controversy regarding their reliability, sensitivity, and applicability. While impressive technological advances have been made in the production of parenteral products, the testing for the quality of these products involves relatively simple procedures. One of the objectives of this book is to critique the adequacy of current methods for sterility, pyrogen, particulate, and leak testing and to review future trends and improved technology in these areas.

The focus of this third edition of *Parenteral Quality Control* is to update the QC practitioner on the ever-changing requirements of various regulatory bodies and to educate the practitioner on new technologies associated with testing methods of the finished product form. Methodologies for performing tests that meet U.S. and European requirements are emphasized.

The late 1990s brought many regulatory changes to the pharmaceutical industry. Not only are inspectors from FDA and European regulatory bodies focusing on quality in manufacturing areas but these regulators are also expecting higher standards, especially with respect to sterility assurance, endotoxin, and particulate control in the quality control laboratory. While the MCA, USP, EP, and FDA all have different expectations, the latter part of the 1990s brought great harmonization where quality control is concerned with the previously mentioned areas of testing. These updates and harmonization effort are discussed in this book.

New to this edition is an expanded discussion of barrier isolation technology, which has become the standard in sterility testing. While no regulations exist for the use of barrier isolation systems, industry expectations exist for qualification of these systems when used for finished-product sterility testing. Regulators are especially concerned with the generation of a false-negative result when surface decontamination is employed with the use of a barrier isolation system. Methods of validation of such a system are discussed. Finally, the chapter on package integrity testing has been updated to educate the reader as to developments in recent technology. Updated references have been added throughout the chapters to provide recent information.

I would like to thank Karim Abdelkader, Ryan Akers, and the marketing department at Baxter Pharmaceutical Solutions LLC for the cover photograph.

Daniel S. Larrimore

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Sterility Testing

INTRODUCTION

Sterility, or freedom from the presence of viable microorganisms, is a strict, uncompromising requirement of an injectable dosage form. Unlike interal administration, parenteral (Greek, *para enteron* = beside the intestine) administration of drugs avoids many of the natural protective defenses of the body. The injection of a product contaminated with living microorganisms would invite a multitude of complications to a potentially immunocompromised patient.

When the term *sterile* appears on the label of a parenteral product, it means that the batch or lot from which the sample originated passed the requirements of the U.S. Pharmacopela (USP) sterility test <71> (or other national compendial sterility test requirement). The USP sterility test provides an estimate of the probable, not actual, sterility of a lot of articles. The actual product administered to a patient has not been tested for sterility. The sterility test is a destructive test; thus, it is impossible to test every item for sterility. This presents a major limitation of the sterility test. Sterility is based on

the results of the testing of a small number of batch samples, assuming that these samples are representative of every article from the batch not tested for sterility. The answer to the question whether the sample is representative of the whole will always be uncertain. Furthermore, another limitation of the sterility test is the finite frequency of accidental (or inadvertent) contamination of one or more samples during the performance of the testing procedures. Regardless of the perfection attempted in the attitudes and techniques involved in sterility testing, accidental contamination will occur with a given percentage of tests conducted. The use of barrier isolation technology (compared to use of a conventional clean room) by the pharmaceutical industry has greatly reduced the chance of accidental contamination that can yield a false positive sterility test.

In light of these and other limitations of the USP sterility test, why is it still a requirement of and enforced by the Food and Drug Administration (FDA) and other regulatory agencies? The most important and obvious reason is to provide some means, albeit small, of end-product testing to protect the consumer from administration of a contaminated injectable product.

An exception to end-product sterility testing involves terminally sterilized large-volume parenterals, which have been exposed to sterilization conditions experimentally validated to assure product sterility well beyond the capability of sterility testing to detect contamination, while products that are terminally sterilized usually have a sterility assurance level (SAL) of at least 10^{-6}. Release of products without end-product sterility testing but based on validation of the sterilization process is called *parametric release* (this will be discussed at the end of this chapter).

While the sterility test does not assure sterility of every article, it does provide the FDA, the manufacturer, and the user with some end-point check that a representative sample of the batch does not disclose the existence of a high proportion of contaminated units in a lot or batch. End-product sterility testing also presents a reliable means of checking the sterility

of a product that has been sterilized by marginal sterilization processes such as aseptic filtration. More discussion of this controversial subject is presented below.

Even if more reliable sterilization methods are used, sterility testing provides an additional means of checking that all facets of the sterilization process were achieved. For example, although steam sterilization is the most reliable sterilization process known, improper loading of the autoclave might prevent adequate steam penetration of some of the product containers, in the batch. A statistically sound sampling procedure (again, a necessary assumption of the sterility test) will select one or more of those improperly exposed containers, and the sterility test will show contamination. Nevertheless, it must be recognized, as it is by the USP, that the sterility test was not designed to ensure product sterility or sterilization process efficacy (1). It simply is a procedure used for sterility control and assurance, along with many other procedures used in manufacturing to assure the sterility of a product.

This chapter will present a thorough and practical analysis of the official testing requirements for sterility, their advantages and limitations, and current adjunct processes and controls to aid in the proper performance and valid interpretation of the sterility test. Also, appropriate focus is placed on the current issues of sterility testing, including retesting of initial test failures, new technology, and sterility testing in the hospital pharmacy. Other review articles on sterility testing include those by Bowman (2), Borick and Borick (3), Beloian (4), Outschoorn (5), and Olson (6). The reader should also be familiar with a published survey of sterility test practices conducted by the Parenteral Drug Association in 1987 (7). This survey can supplement much of the material emphasized in this chapter.

STERILITY AND STERILITY TEST REGULATIONS

Sterility is the most important and absolutely essential characteristic of a parenteral product. Sterility means the complete absence of all viable microorganisms. It is an absolute

term; that is, a product is either sterile or not sterile. Building sterility into a product through meticulously validated cleaning, filtration, and sterilization procedures is more preferable than testing for sterility of a product subjected to marginal or inadequate production processes. The sterility test should never be employed as an evaluation of the sterilization process. Sterility and quality cannot be tested into a product; they can only be components of controlled processes throughout the production sequence (7). The sterility test, however, should be employed as the last of several checkpoints in reaching a conclusion that the production process has removed or destroyed all living microorganisms in the product (2).

The USP chapter <1> on injections states that preparations for injection meet the requirements under "Sterility Tests." After meeting these requirements—that is, all media vessels incubated with product sample reveal no evidence of microbial growth (turbidity)—the tested product may be judged to meet the requirements of the test. If evidence for microbial growth is found, the material tested has failed to meet the requirements of the test for sterility. Retesting is only allowed if there is unequivocal proof that the failed result was due to operator or accidental contamination. The FDA has stringent requirements for sterility retesting.

Evidence for microbial growth is determined by visual evaluation of a vessel containing the product sample in the proper volume and composition of nutrient solution. Provided that the growth conditions are optimal—proper nutrients, pH, temperature, atmosphere, sufficient incubation time, and so on—a single microbial cell will grow by geometric progression* until the number of microbial cells and their metabolic products exceed the solubility capability of the culture medium. Manifestation of this "overgrowth" is visualized by the

* Microbial growth may be characterized by the equation $N = 2^{gt}$, where N is the number of microbial cells, g is the number of generations or replications, and t is the time period during growth. For example, a cell that replicates once every 30 minutes will, after 10 hours, grow to $2^{2\times10} = 2{,}097{,}152$ cells!

appearance of a cloudy or turbid solution of culture medium. A noxious odor may also accompany the turbid appearance of the contaminated medium. The sterility test is a failure when a product generates turbidity in a vessel of culture medium while the same lot of medium without the product sample shows no appearance of turbidity.

Parenteral drug administration was a routine practice in the early 1900s. For example, insulin was discovered in 1921 and was, as it is today, administered by subcutaneous injection. Yet, the first official compendial requirement of sterility testing of drugs administered by the parenteral route did not appear until 1932 in the *British Pharmacopoeia*. Sterility tests were then introduced in the 11th edition of the USP and in the sixth edition of the *National Formulary* (NF) in 1936. During the past 56 years and longer, significant changes and improvements have occurred in the official sterility test requirements; a summary appears in Table 1.1.

Congress passed the Federal Food, Drug, and Cosmetic (FD & C) act in 1938, permitting the FDA to enforce the act. The act recognized the USP and the NF as official compendia to describe the standards of safety, identity, strength, quality, and purity of drugs and the drug dosage form. In 1975, the two compendia were unified. In 1976, the FD & C Act was amended to recognize medical devices as entities to be included in the compendia. Thus, all drug and device products that bypass the gastrointestinal tract on administration to a human being or animal must pass the USP sterility test; this requirement is strictly enforced by the FDA.

Besides the USP/NF official compendia, regulations also exist for two specific groups of pharmaceuticals, the biologics (vaccines, serums, toxins, antitoxins, and blood products) and antibiotics. Sterility tests for biologics and antibiotics are described under Title 21 of the Code of Federal Regulations (8).

In 1978, the final approved regulations of the FDA-authored current good manufacturing procedures (CGMPs) was published. Sterility testing was briefly mentioned under

Table 1.1 Summary of Changes and Improvements in the USP Requirements for Sterility Testing

Year	USP edition	Change or improvement
1936	11th	First year sterility test appeared, applied only to sterile liquids.
1942	12th	Aerobic sterility test in sterile solids and liquids.
		Procedures for inactivation of certain preservatives.
1945	13th	Fluid thioglycollate medium (FTM) introduced for recovery of aerobic and anaerobic bacteria.
		Honey medium introduced for recovery of molds and yeasts.
		Brief description of laboratory area and training of personnel to perform sterility tests.
1950	14th	Incubation temperature of FTM lowered from 37°C to 32–35°C.
		Sabouraud liquid medium (modified) replaced honey medium.
1955	15th	USP Fluid Sabouraud medium replaced the modified Sabouraud medium.
1970	18th	Soybean-casein digest medium replaced.
		Sabouraud medium.
		Membrane filtration sterility test introduced.
		Guidelines included for specific use of biological indicators.
		Expanded sections on describing the area, personnel training, and techniques for performing sterility tests.
1975	19th	Established separate section for membrane filtration procedures.
		Included test procedure for large-volume solutions (≥100 ml).
1980	20th	Section introduced on growth promotion testing using specific indicator microorganisms.
		Section introduced on sterility testing of prefilled disposable syringes.
		Provided guidelines on first and second retests of suspected false-positive tests.

		General expansion and/or elaboration of sections of bacteriostasis, sterility testing of devices, and sterilization.
		Sterilization section contained statement of F_0 = 8 minutes for steam-sterilized articles and D values provided for biological indicators.
1985	21st	Sections on biological indicator paper strips for dry heat sterilization, ethylene oxide sterilization, and steam sterilization.
		Stricter requirements for repeating failed sterility tests.
		Section on basic principles of process validation in the sterility assurance of compendial articles.
		Expansion of information on sterilization by ionizing radiation and filtration.
		Section on definition of a lot for sterility test purposes.
		Reorganization and expansion of section on performance, observation, and interpretation of sterility test results.
		Deletion of procedures for sterility testing of sutures and petrolatum gauze.
1990	22nd	Expansion of bacteriostasis and fungistasis section on use of membrane filtration.
		Further guidance for membrane filtration testing of product having inherent bacteriostatic properties.
		New section on membrane filtration testing procedure for filterable solids.
2000	24th	Addition of *Pseudomonas aeruginosa* and *Staphylococcus aureus* to the bacteriostasis/fungistasis test. New guidance on the use of isolators and sterility testing areas. Addition of the 14 day incubation period for products which are not terminally sterilized by moist heat.

Sect. 211.167, "For each batch purporting to be sterile, there shall be appropriate tests to determine conformance to such requirements." To elaborate on this requirement and to address more specific issues confronted by both industry and the FDA in manufacturing and control of aseptically produced drug products, the FDA published its "Guidelines on Sterile Drug Products Produced by Aseptic Processing" in 1987. These guidelines as they relate to sterility testing are covered in detail below.

SAMPLING FOR STERILITY TESTING

In pharmaceutical manufacture, the sterility of a parenteral product lot is checked by a statistically valid sampling procedure. After years of experience, most manufacturers of parenteral products will sterility test 10 to 20 units of product per lot. The number of units tested may be doubled when the deliverable volume is 1 ml or less. The number of units sampled depends on the number of units in the batch, the volume of liquid per container, the method of sterilization, the use of a biological indicator system, and the good manufacturing practice requirements of the regulatory agency for the particular product. For example, if the batch size is greater than 500 articles, a minimum of 20 units is sampled. If the final batch size is between 100 and 500 articles, then no fewer than 10 of the articles are sterility tested, although there are minimum requirements for sterility testing of biologics. For large-volume parenteral (LVP) products (volume $>$ 100 ml per container), at least 2% of the batch or 10 containers, whichever is less, is sampled. Sampling requirements as specified in the USP and *European Pharmacopeia* (EP) Sterility Test Section are summarized in Table 1.2.

Correct statistical sampling represents a difficult, yet vital, aspect of sterility testing. Realizing that the parenteral product being used by the patient has itself not been tested for sterility, it is absolutely essential that the sampling procedure be as valid and representative of the whole batch as pos-

Table 1.2 Minimum Number of Units Required per Medium for Performance of the USP Sterility Test as a Function of Volume per Test Unit (Quantities for Liquid Articles)

Container content (ml)	Minimum volume taken from each container for each medium	Minimum volume of each medium: Used for direct transfer of volume taken from each container (ml)	Minimum volume of each medium: Used for membrane or half membrane representing total volume from the appropriate number of containers (ml)	No. of containers per medium
Less than 10[a]	1 ml, or entire contents if less than 1 ml	15	100	20 (40 if each does not contain sufficient volume for both media)
10 to less than 50[a]	5 ml	40	100	20
50 to less than 100[a]	10 ml	80	100	20
50 to less than 100, intended for intravenous administration[b]	Entire contents	—	100	10
100 to 500[a]	Entire contents	—	100	10
Antibiotics (liquid)	1 ml	—	100	10

[a] Intended for multiple dose or nonintravenous use.
[b] Intended for single dose or intravenous use.

sible. Realistically, this presents an impossible principle to prove.

Pharmaceutical quality control departments employ sampling plans called *acceptance sampling* for many quality control testing procedures that are not amenable to 100% final testing. Acceptance sampling in sterility testing is based on the establishment of *operating characteristic* (OC) *curves*, which are plots of probability versus percentage contamination. Operating characteristic curves for sample sizes of 10 and 20 units are shown in Figs. 1.1 and 1.2, respectively (9). These curves are drawn from a series of government-sponsored sampling plans called MIL-STD-414 (10). The shape of the curve depends on five criteria:

1. An acceptable quality level (AQL), which is the highest percentage of defective (nonsterile) units that is acceptable
2. An unacceptable quality level (UQL), which is the percentage of nonsterile units for which there is a low probability of acceptance
3. The alpha (α) factor, which is the probability of rejecting a good (sterile) batch
4. The beta (β) error, which is the probability of accepting a bad (nonsterile) batch
5. The sample size

With all criteria (1) through (5) being constant, the slope of the OC curve will become steeper as the sample size is increased. Similarly, with the criteria being constant, the slope of the curve will become steeper as the AQL is decreased or as the UQL is decreased. An example of an OC curve for sampling plans at AQL = 1% for different sample sizes is seen in Fig. 1.3 (11). At a given AQL level, the larger the sample size, the greater is the probability of accepting a sterile lot and rejecting a nonsterile lot. Each pharmaceutical manufacturer for each type of parenteral product assumes a given AQL or rate of contamination, thus fixing the point of reference on the abscissa of the OC curve.

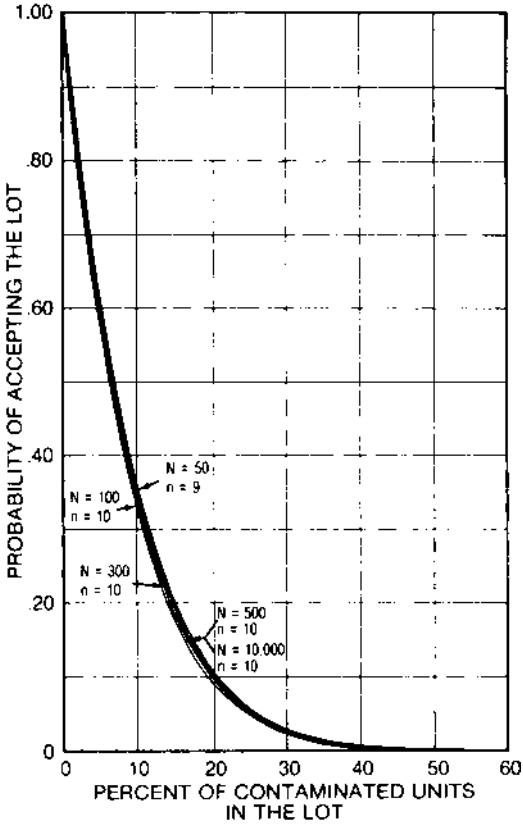

Fig. 1.1 Operation characteristic curves for a sample size of 10 units; n = sample size; N = lot size. (From Ref. 9.)

Sampling plans and concomitant OC curves are prepared on the assumption that the samples are selected at random. By random sampling, it is inferred that any one of the remaining uninspected units of the same lot of product has an equal chance of being selected (12). This is not always easily accomplished. Random sampling often is inconvenient and may not be appreciated by production workers responsible for many other important duties during the production process. Random samples are optimally selected every k^{th} unit, where

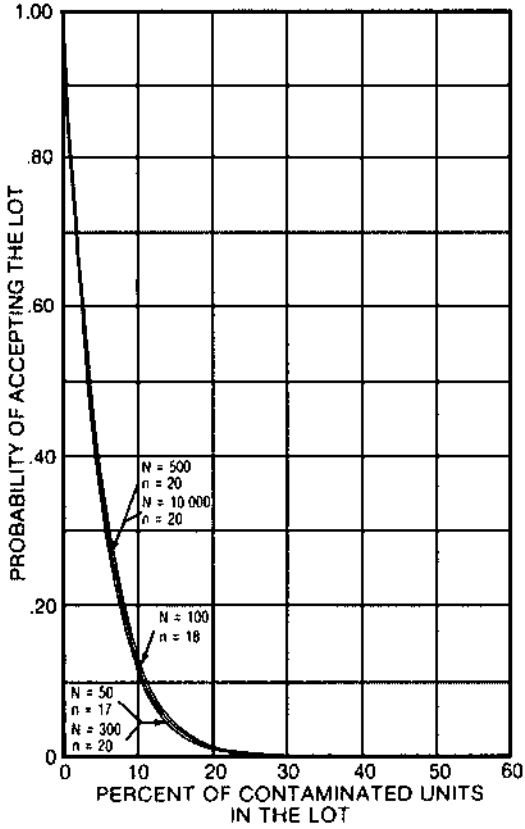

Fig. 1.2 Operating characteristic curves from a sample size of 20 units; n = sample size, N = lot size (From Ref. 9.)

k = the total units in the batch per the number of samples required. For example, if the batch size of an aseptically filled product is 10,000 units and 20 samples are required for the sterility test, then samples are taken every 500 units including the first and last unit filled.*

* Some manufacturers also take sterility test samples immediately after a halted sterile (aseptic) production process has been re-started.

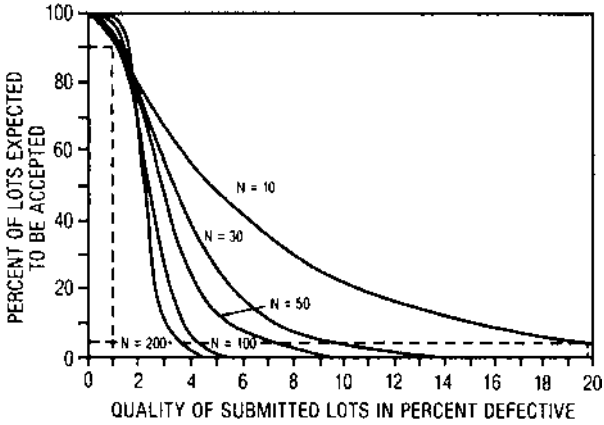

Fig. 1.3 Operating characteristic curve for sampling plans at an acceptable quality level of 1% for different sample sizes N. (From Ref. 11.)

Additional discussion of sampling with regard to its limiting the interpretation of the results of the sterility test is presented in this chapter in the section, "Limitations of the USP/NF Referee Sterility Test."

A major consideration in sampling for sterility testing is proper treatment of the package system to prevent contamination of the sample when it is taken out of the package for testing. For example, parenteral products packaged in ampules, vials, or bottles must be aseptically sampled using sterile materials and aseptic techniques. The neck of the ampule or the surface of the rubber closure must be disinfected with a liquid disinfectant solution before breaking the ampule or penetrating the closure with a needle. Special procedures must be implemented to sample products contained in aluminum foil, paper, or plastic outer bags. For example, bulk solid chemicals sterilized by ethylene oxide prior to aseptic compounding are contained in gas-permeable paper or plastic bags. The chemicals must be sampled by tearing open the package, which is not easy to do because of the potential for accidental contamination. Sutures are contained in glass or aluminum foil enclo-

sures that must be disinfected before the product is removed. Sampling of devices without contaminating the sample also is a very difficult procedure to accomplish. Although the package may be designed to maintain the sterility of the product indefinitely, it is obviously of no value if the inner contents cannot be removed without contaminating the product and interfering with the performance of certain essential tests (3).

CULTURE MEDIA

The USP and EP describe two primary types of culture media to be used in the sterility testing of parenteral products. One type is called fluid thioglycollate medium (FTM), which was introduced by Brewer (13) in 1949. The formulation ingredients of FTM and their basic purpose in the medium are listed in Table 1.3.

FTM provides both aerobic and anaerobic environments within the same medium. Thioglycollate and L-cysteine are antioxidants or reducing agents that maintain anaerobiasis in the lower levels of the culture tube. FTM solution has a two-

Table 1.3 Ingredients of Fluid Thioglycollate medium and Their Purpose

L-Cysteine	0.5 g	Antioxidant
Agar, granulated (moisture content ≤15%)	0.75 g	Nutrient and viscosity inducer
Sodium chloride	2.5 g	Isotonic agent
Dextrose	5.5 g	Nutrient
Yeast extract	5.0 g	Nutrient
Pancreatic digest of casein	15.0 g	Nutrient
Sodium thioglycollate or thioglycollic acid	0.5 g 0.3 ml	Antioxidant
Resazurin sodium solution (1:1000), freshly prepared	1.0 ml	Oxidation indicator
Purified water	QS 1000 ml	

pH after sterilization 7.1 ± 0.2.

color appearance. The pinkish color of the top part of the solution is indicative of the presence of resazurin sodium, an oxygen-sensitive indicator. The pink color should consume no more than one-third of the medium volume. Because of the need for two environments in the same test tube or container, the ratio of the surface to the depth of the medium is very important. To provide adequate depth for oxygen penetration, a 15-ml volume of FTM must be contained in a test tube with dimensions of 20 × 150 mm. A 40-ml volume of FTM is to be contained in 25 × 200 mm test tubes, and 75–100 ml FTM in 38 × 200 mm test tubes.

Devices containing tubes with small lumina are sterility tested using an alternate thioglycollate medium in which the agar and resazurin sodium are deleted. The same medium is used for turbid or viscous parenterals. Without the agar, the medium will not interfere with the viscosity of the product or be as resistant in filling small lumina. Since the medium will be turbid, the presence of a color indicator would not be seen anyway. For oily products, FTM is slightly modified by the addition of 1 ml Polysorbate 80 to 1 liter of the media. Polysorbate 80 serves as an emulsifying agent to permit adequate dispersal of a lipophilic product in a hydrophilic growth medium.

FTM is an excellent medium for the detection of bacterial contamination. Thioglycollate also has the advantage of neutralizing the bacteriostatic properties of the mercurial preservatives. One disadvantage of FTM is that it will not support the growth of *Bacillus subtilis* spores entrapped in solids or material that locates itself in the anaerobic lower portion of the medium (14). *Bacillus subtilis* spores require an environment of high surface tension for normal growth.

The other primary USP/NF culture medium for the sterility testing of parenterals is called soybean-casein digest (SCD) or trypticase soy broth (TSB) medium. The formulation ingredients and their purpose in TSB are shown in Table 1.4.

TSB has a slightly higher pH (7.3 ± 0.2) than FTM (7.1 ± 0.2). TSB replaced Sabouraud medium in the 19th edition of the USP (1970) because TSB was found from experience to

Table 1.4 Ingredients of Trypticase Soy Broth and Their Purpose

Pancreatic digest of casein	17.0 g	Nutrient
Papaic digest of soybean meal	3.0 g	Nutrient
Sodium chloride	5.0 g	Isotonic agent
Dibasic potassium phosphate	2.5 g	Buffer
Dextrose	2.5 g	Nutrient
Purified water[a]	QS 1000 ml	

pH after sterilization 7.3 ± 0.2.
[a] Distilled or deionized water can be used instead of purified water.

be a better medium. It possesses a higher pH and thus was considered a better nutrient for fungal contaminants (15). Fluid Sabouraud, designed to inhibit certain bacteria, was successful in promoting the growth of molds, fungi, and other saprophytes requiring high dextrose content and low pH. TSB, however, promotes growth of fungi and bacteria and is also considered a better medium for than FTM slow-growing aerobic microorganisms.

Other media have been proposed to replace or to substitute for FTM and/or TSB. Abdou (16) found that a dithionite-thioglycollate broth and a peptone liver digest medium were superior to FTM and TSB in growing various strains of bacteria, yeasts, and molds. Concentrated brain heart infusion broth has been suggested as an alternative to FTM and TSB when large-volume parenterals are directly inoculated with culture medium. Table 1.5 lists the formulas of eleven media and reagents potentially used in the sterility testing of parenteral products. While these and other media might be appropriate for certain products or situations, it is highly unlikely that TSB or FTM will be replaced as official USP or EP sterility test media.

Culture media may be purchased in either the dehydrated state or the ready-to-use fluid state. Dehydrated media are less expensive and have a longer shelf life. Strict adherence to the expiration date on the label of premixed culture

Table 1.5 Media and Reagents Potentially Used in Performing the USP/NF Sterility Tests[a]

USP fluid thioglycollate (thio) medium. Use BBL 11260 or Difco 0256.	
Trypticase Peptone (BBL) or Bacto-Casitone (Difco)	15.0g
L-Cysteine	0.5 g
Dextrose (anhydrous)	5.0 g
Yeast extract	5.0 g
Sodium chloride	2.5 g
Sodium thioglycollate	0.5 g
Resazurin (1:1000)	1.0 ml
Agar	0.75 g
Purified water[b]	1.0 liter
Final pH 7.1	
USP soybean-casein digest medium. Use Trypticase Soy Broth (BBL 11768) or Tryptic Soy Broth (Difco 0370).	
Trypticase Peptone (BBL) or Bacto-Tryptone (Difco)	17.0 g
Phytone Peptone (BBL) or Bacto-Soytone (Difco)	3.0 g
Sodium chloride	5.0 g
Dipotassium phosphate	2.5 g
Dextrose	2.5 g
Purified water[b]	1.0 liter
Final pH 7.3	
Polysorbate 80. A suitable grade is TWEEN 80, available from Atlas Chemicals Division, ICI Americas Incorporated.	
Brain heart infusion. Use BBL 11059 or Difco 0037.	
Calf brain, infusion from	200.0 g
Beef heart, infusion from	250.0 g
Gelysate Peptone (BBL) or Proteose Peptone (Difco)	10.0 g
Sodium chloride	5.0 g
Disodium phosphate	2.5 g
Dextrose	2.0 g
Distilled water	1.0 liter
Final pH 7.4	

Table 1.5 Continued

Sporulating agar medium. Use AK Agar No. 2 (Sporulating Agar) (BBL 10912) or Sporulating Agar (Difco 0582).	
Gelysate Peptone (BBL) or Bacto-Peptone (Difco)	6.0 g
Trypticase Peptone (BBL) or Bacto-Casitone (Difco)	4.0 g
Yeast extract	3.0 g
Beef extract	1.5 g
Dextrose	1.0 g
Agar	15.0 g
Manganous sulfate	0.3 g
Distilled water	1.0 liter
Final pH 6.5	
Saline TS, Sterile (USP).	
Sodium chloride	9.0 g
Purified water	1.0 liter
Sabouraud dextrose agar medium. Use BBL 11584 or Difco 0109.	
Dextrose	40.0 g
Polypeptone (BBL) or Neopeptone (Difco)	10.0 g
Agar	15.0 g
Distilled water	1.0 liter
Final pH 5.6	
USP soybean-casein digest agar medium. Use Trypticase Soy Agar (BBL 11043) or Tryptic Soy Agar (Difco 0369).	
Trypticase Peptone (BBL) or Bacto-Tryptone (Difco)	15.0 g
Phytone Peptone (BBL) or Bacto-Soytone (Difco)	5.0 g
Sodium chloride	5.0 g
Agar	15.0 g
Purified water[b]	1.0 liter
Final pH 7.3	
Fluid Sabouraud Medium. Use Sabouraud Liquid Medium (Difco 0382).	
Dextrose	20.0 g
Polypeptone Peptone (BBL) or Neopeptone (Difco)	10.0 g
Distilled water	1.0 liter
Final pH 5.7	

Table 1.5 Continued

USP antibiotic (agar) medium 1. Use BBL 10937 or Difco 0263.	
Gelysate peptone (BBL) or Bacto-Peptone (Difco)	6.0 g
Trypticase Peptone (BBL) or Bacto-Casitone (Difco)	4.0 g
Yeast extract	3.0 g
Beef extract	1.5 g
Dextrose	1.0 g
Agar	15.0 g
Purified water[b]	1.0 liter
Final pH 6.6	
Potato dextrose agar medium	
Potato agar	15.0 g
Glucose	20.0 g
Distilled water q.s.	1.0 liter
pH 5.6 ± 0.2	

[a] Sterilize in an autoclave at 121°C (15 lb pressure) for 15 minutes unless otherwise indicated. If commercial preparations are not available, equivalent preparations may be used.
[b] Distilled or deionized water can be used instead of purified water.

media tubes must be obeyed, provided that the proper storage conditions (usually refrigeration) have been met.

Preparation of sterile fluid culture media from dehydrated media is a relatively simple process. The label of each container of medium describes the procedure for preparation. Basically, the procedure involves (a) weighing the appropriate amount of medium per liter of fluid desired, (b) adding water to the compounding vessel to the desired volume, (c) slowly adding the culture medium while stirring the solution, (d) applying heat and stirring until the medium is completely dissolved, and (e) sterilizing the medium in bulk or after filling into test tubes or other containers by steam heat under pressure by a validated sterilization cycle. Before discarding contaminated culture media, they must be again sterilized by steam under pressure before pouring the fluid into a drainage system and washing the containers.

Table 1.6 Formulations of Various Diluting Fluids Used with the Membrane Filtration Test Method

Diluting Fluid A	
Peptic digest of animal tissue	1.0 g
Distilled water	1.0 liter
pH 7.1 ± 0.2	
Diluting Fluid D	
Peptic digest of animal tissue	1.0 g
Polysorbate 80	1.0 ml
Distilled water	1.0 liter
pH 7.1 ± 0.2	
Diluting Fluid A modified	
Peptic digest of animal tissue	1.0 g
Ascorbic acid	10.0 g
Distilled water	1.0 liter
Diluting Fluid E	
Isopropyl myristate	100.0 ml
Water extract pH not less than 6.5	
Medium K	
Peptic digest of animal tissue	5.0 g
Beef extract	3.0 g
Polysorbate 80	10.0 g
Distilled water	q.s. 1.0 liter
pH 6.9 ± 0.2	

When membrane filtration is used for the sterility test, a diluting fluid must be used to rinse the filtration assembly to ensure that no microbial cells remain anywhere but on the filter surface. The diluting fluid may also be used to dissolve a sterile solid prior to filtration. Some examples of diluting fluid formulas are listed in Table 1.6. Diluting fluids are intended to minimize the destruction of small populations of vegetative cells during the pooling, solubilizing, and filtering of sterile pharmaceutical products (17).

TIME AND TEMPERATURE OF INCUBATION

No ideal incubation time and temperature condition exists for the harvesting of all microorganisms. Most organisms grow

more rapidly at 37°C than at lower temperatures. However, a temperature of about 23°C may reveal the presence of some organisms that might remain undetected if incubations were done at higher temperatures (18). Pittman and Feeley (19) demonstrated that temperatures of 22°C and 30°C were more favorable for the recovery of yeasts and fungi in FTM than a temperature of 35°C. The Division of Biologics Standards of the National Institutes of Health discovered that a pseudomonad contaminant in plasma grew in FTM at 25°C, but was killed at 35°C (2). As a result of this finding, the incubation temperature range of FTM was lowered from 32–35°C to 30–35°C as required by the USP/NF (20th edition).

The current time and temperature incubation requirements of the USP sterility test are found in Table 1.7. Incubation in TSB is accomplished at 20–25°C because of favorable growth of fungal and slow-growing aerobic contaminants at this temperature range. The time of incubation for sterility testing by membrane filtration (MF) is 7 or 14 days.

Findings by Bathgate et al., suggest that a 7-day incubation period is inappropriate for detecting a potentially contaminated unit. The authors suggest that at least 14 days is required to significantly increase the chance of detecting most contamination in the sterility test and cite several examples for which certain tests were negative after 7 days of incubation. Some contaminants did not begin to show signs of visible growth until the 10th day of incubation. The article reports additional problems when ethylene oxide was used for sterilization or when an antimicrobial preservative was employed in the drug product. The data in this article also suggest that there is no significant advantage of the MF test when compared to the direct transfer (DT) test (105).

Others disagree with Bathgate et al. (105), citing the already low chance of the compendial sterility test to detect a contaminated unit in a batch. These authors cite many reasons why a 7 day incubation period is adequate to detect contamination in a sterility test (106).

No doubt the debate to continue the sterility test will continue for years. The time and temperature incubation require-

Table 1.7 Time and Temperature Incubation Requirements of the USP Sterility Test

Medium	Test procedure	Time (days)[a]	Temperature (°C)	Sterilization method
FTM	Direct transfer	14	30–35	Steam or aseptic process
	Membrane filtration = 100 ml	7	30–35	Terminal moist heat sterilization
	Membrane filtration = 100 ml	14	30–35	Aseptic process
TSB	Direct transfer	14	20–25	Steam or aseptic process
	Membrane filtration = 100 ml	7	20–25	Terminal moist heat sterilization
	Membrane filtration = 100 ml	14	20–25	Aseptic process

[a] Minimum number of incubation days. Additional incubation time may be required if the product is conducive to producing a "slow-growing" contaminant.

Table 1.8 Time and Temperature Incubation Requirements of the EP Sterility Test

Medium	Test procedure	Time (days)[a]	Temperature (°C)	Sterilization method
FTM	Direct transfer	21 (14 + 7)	30–35	Steam or aseptic process
	Membrane filtration = 100 ml	7[b]	30–35	Terminal moist heat sterilization
	Membrane filtration = 100 ml	14	30–35	Aseptic process
TSB	Direct transfer	21 (14 + 7)	20–25	Steam or aseptic process
	Membrane filtration = 100 ml	7[b]	20–25	Terminal moist heat sterilization
	Membrane filtration = 100 ml	14	20–25	Aseptic process

[a] Minimum number of incubation days. Additional incubation time may be required if the product is conducive to producing a "slow-growing" contaminant.
[b] A 7-day incubation period is permissible only when authorized or dictated in the European Medicines Evaluation Agency (EMEA) submission. In general, a 14-day incubation period is required for all products that are required to meet the EP sterility test.

ments of the USP and EP tests are summarized in the Tables 1.7 and 1.8, respectively.

The incubation time requirements of the sterility test must be sufficiently long to account for the variable lag time characteristic of the growth curve of most microbial forms. A typical growth cycle for bacteria is seen in Fig. 1.4. At the beginning of the cycle, corresponding to the time at which the test sample is combined with the culture medium, there exists a lag time phase. The length of this time depends on the rapidity of the microbial cell to adapt to its "new" environment. Usually, the lag phase lasts no longer than a few hours, but the possibility is always present that a resistant spore form, a slow-growing contaminant, or a microorganism with an extra

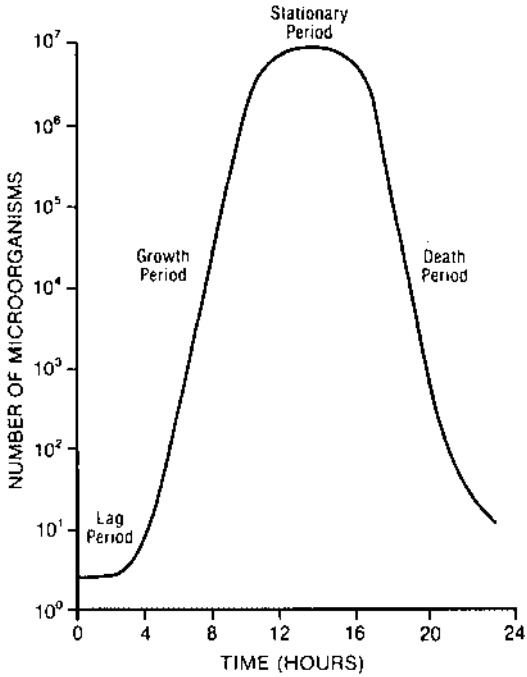

Fig. 1.4 Typical growth-and-death cycle for bacteria.

long lag phase owing to damage caused in the sterilization process may be part of the test sample. Sufficient incubation time must be allowed for the microbial form to overcome its own resistance to grow in the FTM or TSB environment. However, once the lag phase is completed, the growth phase is exponential. Most contaminated samples will show evidence of contamination within 24 to 48 hours due to the meteoric growth of microorganisms.

For the direct transfer method, it is possible that a physiochemical incompatibility between the product inoculum and the culture medium might exist, resulting in a precipitate or turbid reaction not indicative of microbial growth. Should this occur, the appropriate action to take is to transfer an aliquot (usually 1 ml) of the suspension to fresh culture medium on the days 3 to 7 (for the USP test) and day 14 (for the EP test) after the test was started and incubate both the original and the new media for a total of, but not less than, 7 additional days. Thus, in all cases, the original and aliquot vessels would be incubated for 14 days (for the USP test) or 21 days (for the EP test). Incubation time and temperature requirements are shown in Table 1.7 and 1.8 for the USP and EP sterility tests, respectively.

Incubation time and temperature requirements for sterility tests conducted under the auspices of various authorities are basically similar to those of the USP presented in Table 1.7, although subtle differences in the sterility test do exist for the EP sterility test (see Table 1.8). The importance of time of sampling in hospital intravenous admixture sterility testing was discovered by DeChant et al. (20). They found that no more than 1 hour should transpire between preparation of intravenous admixtures and sampling of the admixture for conducting a sterility test. If longer time periods are permitted, microorganisms, if introduced during the admixture preparation period, may be inhibited from reproducing because of bactericidal activity of certain intravenous solutions, such as dextrose 5% in water.

STERILITY TEST METHODS

The USP and EP sterility tests specify two basic methods for performing sterility tests, the direct transfer (DT) or direct inoculation method and the MF method, with a statement that the latter, when feasible, is the method of choice. In fact, in some cases, membrane filtration may be the only possible choice. Suggested standard operating procedures for performing both methods are given at the end of this book as Appendices I and II.

Direct Transfer Method

The DT method is the more traditional sterility test method. Basically, the DT method involves three steps:

1. Aseptically opening each sample container from a recently sterilized batch of product
2. Using a sterile syringe and needle to withdraw the required volume of sample for both media from the container
3. Injecting one-half of the required volume sample into a test tube containing the required volume of FTM and the other half volume of sample into a second test tube containing the required volume of TSB

The DT method is simple in theory, but difficult in practice. The technician performing the DT test must have excellent physical dexterity and the proper mental attitude about the concern for maintaining asepsis. The demand for repetition in opening containers, sampling, transferring, and mixing can potentially cause fatigue and boredom, with a subsequent deterioration in operator technique and concern. As this occurs, the incidence of accidental product sterility test contamination will increase.

The USP and EP tests require a minimum volume of sample per container volume to be transferred to a minimum volume of each culture medium. Table 1.9 lists these volume requirements. The sample volume must be a sufficient representation of the entire container volume and the volume,

Table 1.9 Volume Requirements of the Direct Transfer Sterility Test

Container content (ml)	Minimum volume of product (ml)	Minimum volume of medium (ml)
10 or less	1 (or total contents if less than 1 ml)	15
10–50	5	40
50–100	10	80
100–500	One-half contents	N/A
>500	500	N/A
Antibiotics (liquid)	1 ml	N/A

N/A, not applicable.

of medium must be sufficient to promote and expedite microbial growth, if present. Adequate mixing between the sample inoculum and the culture medium must take place to maximize interaction and facilitate microbial growth.

Membrane Filtration Method

The MF sterility test became official in the 18th edition of the USP in 1970. It has since become the more popular and widely used method over the DT method and, when feasible for pharmacopeial articles, should be preferred. Specific application of the MF sterility test method has been the subject of many publications, for example, those concerned with the sterility testing of antibiotics (17), insulin (21), and LVPs (22).

The successful employment of this technique requires more skill and knowledge than that required for the DT method. Five basic steps are involved in the use of the MF sterility test method:

1. The filter unit (Fig. 1.5) must be properly assembled and sterilized prior to use.
2. The contents of the prescribed number of units are transferred to the filter assembly under strict aseptic conditions.

Fig. 1.5 Set-up of the classic membrane filtration sterility test apparatus. (Courtesy of Eli Lilly Co., Indianapolis, Indiana.)

3. The contents are filtered with the aid of a vacuum or pressure differential system.
4. The membrane is removed aseptically and cut in half.*
5. One-half of the membrane is placed in a suitable volume (usually 100 ml) of FTM, and the other membrane half is placed in an equal volume of TSB.

A suitable membrane filter unit consists of an assembly that facilitates the aseptic handling of the test articles and allows the processed membrane to be removed aseptically for transfer to appropriate media or an assembly by which sterile media can be added to the sealed filter and the membrane incubated in situ. A membrane suitable for sterility testing has a rating of 0.45 μm and a diameter of approximately 47 mm.

* The USP gives the option of using two "whole" membranes, one for each medium, or cutting a single membrane.

These membranes have hydrophobic edges or low product-binding characteristics that minimize inhibitory product-residue, and it is this residue that interferes with requirements of the validation test for bacteriostasis and fungistasis. For products that do not contain inhibitory substances, membranes without hydrophobic edges can be used, but should be wetted prior to testing.

The cleaning, assembly, sterilization, and final connections involved in the preparation of the membrane filtration equipment are described in Appendix III. Complete description and application of membrane sterility test methods for antibiotics, nonantibiotics, and ophthalmics may be studied using the Millipore Application Manual AM201 (Millipore Corp., Bedford, MA 01730).

The membrane filtration technique has been further developed by the Millipore Corporation, and the Steritest® system has become widely used when the MF technique is employed. The Steritest system is essentially the same as the conventional MF system, but greatly reduces the chance of accidental contamination of the test by keeping the sterility test system as closed as possible.

The MF method offers at least five advantages over the use of the DT method:

1. Greater sensitivity (23).
2. The antimicrobial agent and other antimicrobial solutes in the product sample can be eliminated by rinsing prior to transferring the filter into test tubes of media, thereby minimizing the incidence of false-negative test results.
3. The entire contents of containers can be tested, providing a real advantage in the sterility testing of large-volume parenterals and increasing the ability to detect contamination of product lots containing very few contaminated units.
4. Low-level contamination can be concentrated on the membrane by filtering large volumes of product. This results in faster reporting of test results since MF requires only

7 days incubation (for most terminally sterilized products).
5. Organisms present in an oleaginous product can be separated from the product during filtration and cultured in a more desirable aqueous medium.

Conversely, the MF method presents two major disadvantages compared to the DT method:

1. There exists a higher probability of inadvertent contamination in manual operations because of the need for greater operator skill and better environmental control in disassembling the filtration unit and removing, cutting, and transferring the membrane. (Newer systems such as the Steritest have eliminated this disadvantage.)
2. The method is unable in differentiate the extent of contamination between units, if present, because all product contents are combined, filtered through a single filter, and cultured in single test tubes. Also, if accidental contamination has occurred, rather than this being detected in one or more vessels of the DT method, it manifests itself in the only container used per culture medium.

Interpretation of Results

If there is no visible evidence of microbial growth in a culture medium test tube, after subjecting the sample and medium to the correct procedures and conditions of the USP and EP sterility test, it may be interpreted that the sample representing the lot is without intrinsic contamination. Such interpretation must be made by those having appropriate formal training in microbiology and having knowledge of several basic areas involved in quality control sterility tests:

1. Industrial sterilization methods and their limitations
2. Aseptic processing
3. Statistical concepts involved in sampling lots for representative articles
4. Environmental control procedures used in the test facility

If microbial growth is found or if the sterility test is judged to be invalid because of inadequate environmental conditions, the sterility test may be repeated. However, this introduces a controversial and somewhat complicated subject.

STERILITY RETESTING

Sterility retests have been allowed by the USP since sterility testing became a USP requirement (XI edition, 1936), but only when the USP XX edition (1980) was published was there specific definitions of first and second sterility retests. While sterility retesting is allowed per the Code of Federal Regulations (CFR), retesting without just cause is no longer allowed per USP as of the eighth supplement, released in May 1998.

The FDA has repeatedly reaffirmed that it supports the USP position provided that industry shows due diligence in their investigations of initial sterility test failures. Avallone (24) wrote an FDA position paper in 1986 pointing out limitations of the sterility test and, in fact, summarizing that "a positive test result could indicate that the sample of product tested was truly contaminated, while a negative test result does not really mean that much, or even that the sample tested was truly sterile." He describes the pharmaceutical industry as having different levels of quality philosophy regarding sterility tests. On one end there are manufacturers who recognize the many limitations of aseptic processing and sterility testing, so that if there is a sterility test failure, the batch is rejected. On the other end, there are manufacturers who will do everything to justify the release of a product that fails an initial sterility test. Avallone goes on to discuss all the various activities that a firm should consider when investigating an initial sterility test failure and the decision-making process that should be undertaken to release the lot of product. His final statement in this article is worth repeating: "The management of a firm truly committed to quality has little if any problem in the interpretation of sterility test results." Thus, sterility retesting and investigation of initial sterility test fail-

ures should be done with the highest degree of diligence and responsibility on the part of high-level management of the parenteral industry.

FDA GUIDELINES ON STERILITY TESTING

The June 1987 FDA Guideline on Sterile Drug Products Produced by Aseptic Processing contains a fair amount of direction regarding conductance, evaluation, limitations, interpretation, and retesting requirements of the USP sterility test. The testing laboratory environment should employ facilities and controls comparable to those used for the filling and closing operations (e.g., Class 100 air conditions for critical operations when a sterile product is exposed to the environment). The limitations of the USP sterility test (as discussed on pp. 50–58) cause the FDA considerable concern with respect to sampling plans and any positive test result that may occur. In investigation of sterility test failures/positive test results, the guidelines state: "When persuasive evidence showing laboratory error is absent, or when available evidence is inconclusive, firms should err on the side of safety and batches should be rejected as not conforming to sterility requirements." This statement has caused much consternation among quality control (QC) groups in the pharmaceutical industry because assurance of sterility is so difficult to prove with absolute certainty.

Investigations of sterility test failures should consider every single factor related to the manufacture of the product and the testing of the product sample. Tables 1.10–1.12 show representative lists of factors to be investigated by QC both in the manufacturing areas and in the sterility test laboratory to determine how a sterility test failure could have occurred. Most of the time, there is no concrete conclusive evidence pinpointing where the contamination occurred; thus, QC must make a decision based on philosophical positions and retrospective history of the manufacturing and sterility test areas.

Table 1.10 Manufacturing Quality Control Checklist for Investigating Sterility Test Failures (Aseptically Filled Products)

Item Code: _____ Investigated by: _____
Lot No.: _____ Disposition Date: _____

____Line validation by media fill
____Sterilization records of primary packaging components
____Sterilization records of product contact or component contact equipment
____Environmental monitoring data
    ____viable particle counts
    ____nonviable particle counts
    ____surface testing results
    ____pressure differentials
        ____log of room differentials
        ____verification of laminar flow gauges in aseptic manipulation zones
    ____temperature
    ____humidity
____Bioburden of product-contact utilities
    ____water
    ____compressed gases (i.e., nitrogen, air)
    ____clean steam
    ____raw materials
____HEPA filter certifications for filters of the aseptic manipulation zone
____Sanitization logs
____Sterilizing filter integrity test results for filters servicing the following product contact utilities:
    ____compressed gases
    ____vent filters (vacuum)
____Product sterilizing filter integrity test
____Preventive maintenance and calibration records for critical parameter instruments of:
    ____autoclaves
    ____freeze dryers
    ____Strunck tunnels
    ____hot-air ovens

Table 1.10 Continued

____Corrective maintenance records and/or a physical inspection of the equipment used to manufacture the lot in question, as applicable (i.e., gaskets, joints, valves, piping, etc.)
____Manufacturing ticket
____Deviation reports, if any
____Incoming sterility tests of purchased sterilized primary packaging components
____Vendor issues form Purchased Materials QC audits
____Operator training records
  ____personal broth test
  ____gowning training
  ____aseptic technique training
  ____operator garment monitoring
____Any other manufacturing-related parameter that might have an impact on product sterility/integrity

Table 1.11 Manufacturing Quality Control Checklist for Investigating Sterility Test Failures (Terminally Sterilized Products)

Item Code: _____ Investigated by: _____
Lot No.: _____ Disposition Date: _____

____Product sterilizing filter integrity tests
____Preventive maintenance and calibration records for autoclave(s) used
____Product terminal sterilization records
  ____charts
  ____loading sheets

Table 1.12 Quality Control Sterility Laboratory Checklist for Investigating Sterility Test Failures

Item Code: _____ Investigated by: _____
Lot No.: _____ Disposition Date: _____

____Test information
- ____medium in which the growth was observed
- ____ID of the isolate
- ____number of lots or sections tested in the same test run
- ____other tests showing contamination from the same test run
- ____analyst number of the individual performing the test
- ____hood in which the test was performed

____Sterilization records and BI results pertaining to the sterilization of the equipment and media used to perform the sterility test of the lot in question

____Environmental monitoring data
- ____viable particle counts
- ____analyst garment monitoring

____Test controls
- ____negative controls of the media used to perform the test of the lot/section in question
- ____manipulative negative control
- ____equipment sterility check
- ____media preparation and growth promotion records

____Sanitization logs for
- ____chemical sanitization of room
- ____peracetic acid sanitization of room

____HEPA filter certifications for:
- ____incoming air to room
- ____laminar flow hoods

____Analyst training records
- ____analyst certification
- ____gowning procedure
- ____aseptic technique training

____Lab retest data
- ____by item, by line, by presentation

The FDA aseptic guidelines indicate that persuasive evidence of the origin of the contamination should be based on the following:

1. The identification of the organism in the sterility test (genetic typing may be useful or required)
2. The laboratory's record of tests over time
3. Monitoring of production area environments
4. Product presterilization bioburden
5. Production record review
6. Results of sterility retest

Thorough and complete review of all these data should enable reviewers to determine the actual, or certainly probable, origin of the organism contaminating the sterility test sample. In addition to the discussion below, Avallone (24) and Lee (25) have written detailed articles on the evaluation and investigation of initial sterility test failures.

Identification of the Organism in the Sterility Test

Not only the genus, but also the species of the isolated organism will provide invaluable information concerning the organism's habitat and its potential resistance to the product formulation and sterilization methods. If the organism is one normally found on people, then the investigation can focus on employee hygiene, washing and gowning techniques, and aseptic techniques. Identification of the organism can be compared to historical microbial databases for the manufacturing and testing areas to assess probabilities of the origination of the organism. Obviously, if the organism identified had been isolated before in the production area, but never in the testing area, then the production area would be implicated as the source of the organism, and the test would be judged as a true sterility test failure. Identification of the organism allows the manufacturer to perform further testing to determine if the organism is sensitive to the product formulation, particularly if the product contains an antimicrobial preservative. If an organism that was isolated from a product that was terminally sterilized and that had resistance to terminal sterilization is

proven to be below the microbial reduction produced by the sterilization cycle, then it can reasonably be deduced that the organism did not originate from the product. Knowledge of whether the organism identified is an aerobe or anaerobe would be important if the product were one that contained antioxidants or was overlayed with nitrogen. For example, if the organism were a strict anaerobe and the product was flushed with nitrogen prior to sealing, then it must be strongly suspected that the organism originated during the manufacturing process and was protected by the nitrogenation of the product.

The Laboratory's Record of Tests Over Time

The FDA finds it normal for a sterility testing laboratory to have an initial positive sterility test failure of less than 0.5% of all sterility tests. Therefore, if the laboratory shows a failure rate higher than 0.5%, then problems must exist in the laboratory, production areas, or both. Trends of sterility test failures should be noted, and rates of sterility test failure should be grouped according to product type, container type, filling line, and degree of manual manipulation. If a product is terminally sterilized and the sterility test failure rate shows an upward trend, then problems in the testing environment or personnel can be suspected and action taken to eliminate the problem. Conversely, upward trends in test failure of a product or line of products manufactured aseptically can indicate production problems that should be investigated. Not only monitoring of failure rates as a function of time, but also monitoring of environmental test data in both the production and testing areas can provide important information for follow-up to correct potential sources of contamination and keep the false-positive failure rates extremely low.

Monitoring of Production Area Environments

Every manufacturing area should have a thorough and complete record of environmental monitoring data obtained daily as the area is being used. Trend analysis should be done on

air-monitoring data (agar settle plates, viable air sampling, particulate matter analyses) and surface monitoring (Rodac, swab). Also, monitoring data of production personnel should be closely scrutinized to ensure that personnel are practicing good aseptic techniques.

Recent FDA inspections have resulted in 483 citations either because the manufacturer either failed to have sufficient environmental data or because such data were not used properly in assessing the acceptability of a lot that failed the sterility test initially. For example, environmental data showed contamination on the line used to fill a product that subsequently failed a sterility test, but such contaminants on the line were different from that found in sterility test failure. The firm repeated the sterility test and released the lot on the basis that the contaminant in the initial sterility test failure did not come from the manufacturing area since it was different from contaminants found in environmental testing of the area. The FDA concluded that just because a contaminant found in a sterility test failure was not specifically identified in the manufacturing environment, it does not preclude the contaminant's presence in that manufacturing area. The firm's environmental monitoring data indicated the presence of a problem, and the firm did not, according to the FDA, react responsibly toward this problem. Unequivocal proof must exist to invalidate an initial sterility test positive, and the FDA will heavily scrutinize any lot that is released that has failed a sterility test. A thorough investigation with sound scientific proof should be performed before repeating or releasing a batch of material that has failed the sterility test.

Product Presterilization Bioburden

The bioburden of each lot of product should be known before such product is sterilized either by terminal sterilization or by aseptic filtration. Both methods have limits of sterilization. A sterilizing grade filter with a nominal pore size of 0.22 μm is usually capable of retaining a bioburden of no more than 10^7

CFU per cm^2 as a function of filter surface area, while terminal sterilization is typically capable of a 10^6 log reduction of bioburden. Trend analysis of product bioburden will determine if an upward trend in bioburden might be occurring and if this might be a contributor if sterility test failures also are showing an upward trend over time.

Production Record Review

All records of producing a batch of product should be reviewed (see Tables 1.10–1.12). Any one of these records showing an aberrant result (e.g., high particulate counts, lack of proof that a certain operation was done, a sterilization run that had problems, etc.) could help in determining the point at which this aberrancy had an impact on product sterility.

Results from all the above investigations should be collectively reviewed by competent and experienced personnel and reviewed by upper management, preferably a representation from a variety of departments—QC, production, technical services, quality assurance (QA)—before a final decision is made regarding the acceptance or rejection of a batch of product that initially failed a sterility test.

It is rare that a determinant cause can be identified for many sterility test failures, and it is an FDA expectation that a specific cause be identified before a sterility test can be repeated. Some reasons to invalidate a sterility test may be failed environmental monitoring in the sterility testing room or isolator, a negative control that is positive after incubation, cracked media containers, poor aseptic technique during the sterility test, improper sanitization or sterilization of the sterility testing area, defective stoppers and/or seals on sterility samples, and so on.

The lot may only be released if a definitive reason is discovered to invalidate the initial failure. Only then can the test be repeated and the lot released. According to USP XXV, the test may only be repeated if the positive sterility test result can "without a doubt be ascribed to faulty aseptic techniques

or materials used in conducting the sterility testing procedure".

STERILITY TESTING OF DIFFERENT STERILE PRODUCTS

The USP describes the sterility test procedures to be followed for all types of sterile products; in addition, biologics, human antibiotics, and veterinary biologics must comply with federal regulations (9CFR113.26). Test procedures for the DT to test media are given for the following five types of products from USP:

1. Nonfilterable Liquids
2. Ointments and oils insoluble in isopropyl myristate
3. Solids to test media
4. Purified cotton, gauze, surgical dressings, sutures, and related articles
5. Sterilized devices

Test procedures for using the MF technique are specified for the following nine types of products:

1. Liquids miscible with aqueous vehicles
2. Liquids immiscible with aqueous vehicles, less than 100 ml per container
3. Ointments and oils soluble in isopropyl myristate
4. Prefilled syringes
5. Solids for injection other than antibiotics
6. Antibiotic solids for injection
7. Antibiotic solids, bulks, and blends
8. Sterile aerosol products
9. Devices with pathways labeled sterile

For a complete description of these test procedures, refer to the appropriate section of the USP or EP. In the following discussion, each test procedure is summarized and additional information not found in the USP description is provided to

enhance the reader's understanding and appreciation of the procedure.

Direct Transfer

Nonfilterable Liquids

Summary of procedure: Agitate the containers and aseptically withdraw, from a sufficient number of units, the volumes of medium indicated. Mix each test specimen with the appropriate medium, but do not aerate excessively. Proceed to add each of the contents to TSB and FTM, respectively.

Commentary: The risk of inadvertent contamination is at its greatest during this process. Strict aseptic technique must be practiced to minimize this risk. Also, it must be realized that a finite probability exists that the pipet or syringe and needle may themselves not be sterile.

The USP cautions against excessive mixing of the test sample and the medium. This is especially true for FTM because of the need to preserve the efficacy of the thioglycollate antioxidant, which maintains anaerobiasis in the upper part of the vessel.

At least 14 days (for the USP test) or 21 days (for the EP test) are required to ensure that any microbial contaminants, if present, have been given sufficient time to adapt to the FTM or TSB environment and to begin to thrive and reproduce.

Ointments and Oils Insoluble in Isopropyl Myristate

Summary of procedure: 100 mg (or the entire contents if less than 100 mg) from each of 10 containers are aseptically transferred to a flask containing 200 ml of a sterile aqueous vehicle. Then, 20 ml of this mixture is mixed with 200 ml of FTM and incubated for not less than 14 days (for the USP test) or 21 days (for the EP test). The entire process described above is repeated with another 10 containers using TSB as the medium.

Commentary: Two key facets of this procedure are (a) employing strict aseptic technique in the two transfer processes for each medium, and (b) choosing the correct dispersing agent in the aqueous vehicle that both adequately disperses the oil or ointment homogeneously in the vehicle and, in the concentration used, has no antimicrobial capacity in and of itself. The most commonly used dispersing agents are surface-active agents, such as Polysorbate 80 and Triton X-100, dissolved in water. Some feel, however, that Triton X-100 exerts an antimicrobial effect.

Solids to Test Media

Summary of procedure: Transfer a quantity of product in the form of a dry solid (or prepare a suspension of the product by adding sterile diluent to the immediate container) in the quantity directed in the USP. Transfer the material so obtained to 200 ml of FTM and mix. Repeat the above procedure for TSB. The incubation time is again not less than 14 days (for the USP test) or 21 days (for the EP test).

Commentary: Most sterility testing facilities prefer reconstituting the dry sterile solid with sterile water for injection. The chance of accidental contamination is greatly enhanced because of the extra manipulations involved in reconstituting and then withdrawing the fluid sample for transfer. The adherence to strict aseptic technique cannot be overemphasized.

Purified Cotton, Gauze, Surgical Dressings, Sutures, and Related Articles

Summary of procedure: 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 incubate the test for not less than 14 days (for the USP test) or 21 days (for the EP test).

Commentary: If the entire article is too large to be transferred intact to culture media, a suitable portion or the innermost part of the article is tested and assumed to be representative of the entire article. The rationale for selecting the innermost part is the fact that this part is the most difficult area for steam or gas to penetrate during terminal sterilization. Strict aseptic technique must be followed when performing the above test to avoid inadvertent contamination of the test medium. Since most devices are packaged in paper or Tyvek, which is permeable to gas sterilants, the use of isolators to test devices is often not feasible because of the gas sterilization system associated with them.

Sterilized Devices

Summary of procedure: 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 incubate the test.

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.

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.

In all cases, DT items must incubate for a total of not less than 14 days (for the USP test) or 21 days (for the EP test).

Commentary: If the entire article is too large to be transferred intact to culture medium, a suitable portion or the innermost part of the article is cut out and assumed to be repre-

sentative of the entire article. The rationale for selecting the innermost part is the fact that this part is the most difficult area for steam or gas to penetrate during terminal sterilization. Strict aseptic technique must be followed when performing the above test to avoid inadvertent contamination of the test medium. Again, since most devices are packaged in paper or Tyvek, which is permeable to gas sterilants, the use of isolators to test devices is often not feasible because of the gas sterilization system associated with them.

Membrane Filtration

Liquids Miscible with Aqueous Vehicles

Summary of procedure: At least 20 or 40 containers of product are used. Sufficient volumes required for both media are transferred aseptically into the membrane filter funnel or withdrawn from the container using the Millipore Steritest system. Vacuum or pressure is applied, and the solution is filtered. The membrane is removed aseptically and cut in half; one half is placed in 100 ml of FTM, while the other half is placed in 100 ml of TSB. In the case of the Steritest system, the filter is contained within the chamber that is incubated. Incubation is carried out for not less than 7 days (for products that are terminally sterilized by moist heat) or 14 days (for products that are required to meet EP standards or are not terminally sterilized by moist heat).

For (a) LVP solutions, (b) 50 ml to less than 100 ml for intravenous use, or (c) between 100 ml and 500 ml, the entire contents of 10 containers are aseptically transferred and filtered through each of two filter assemblies, or if only one filter assembly is used, or 20 containers are emptied or withdrawn from the container using the Millipore Steritest system. For LVP solutions with volumes greater than 500 ml, at least 500 ml are transferred from each of 10 containers through each of two filter as-

semblies or from each of 20 containers if one filter assembly is used. Then, the membrane is removed using sterile forceps, cut in half with sterile scissors, and the halves aseptically added to 100 ml of FTM and TSB, respectively.

With the high usage frequency of total parenteral nutrition solutions in hospital practice, many LVPs are now available containing high concentrations of dextrose. These and other highly viscous solutions are filtered through several filter assemblies since one assembly will not permit the passage of the entire contents of a viscous solution. However, the total volumes and number of containers per medium remain the same as required for nonviscous solutions. Half of the total number of membranes used are incubated in each medium.

Commentary: While the MF method offers distinct advantages over the DT method, the risk of extraneous contamination is greatly increased because of the manipulations additional to those employed in conducting the sterility test by DT. Thus, extreme precautions must be followed in all the techniques involved in the MF method. Negative controls are especially recommended with the above methodology. Use of apparatuses such as Steritest and isolation systems have greatly reduced the number of manipulations required by the operator in using the MF technique. These systems thus have greatly helped minimize adventitious contamination and strengthened the advantages of the MF technique.

In transferring the container contents into the membrane filter funnel, great care must be used to avoid squirting solution directly onto the filter. Also, since this method is used to sterility test small-volume multidose parenterals containing antimicrobial preservatives, the membrane must be rinsed three times with USP Diluting Fluid A (100 ml) to ensure that the entire solute content has been washed through the membrane. The MF method is an excellent technique for the sterility testing of LVP

solutions because low levels of contaminants in these dilute solutions are concentrated together on the surface of one or two filters. If only the direct transfer method were available, even a representative sample of LVP added to culture media would contain an insufficient number of microbial cells to harvest under the best of incubation conditions.

Liquids Immiscible with Aqueous Vehicles (Less than 100 ml per Container)

Summary of procedure: The required volume from 20 containers is transferred aseptically directly into one or two separate membrane filter funnels. After filtration via vacuum, the membrane is cut in half using the aseptic procedure already described and is incubated in 100 ml each of FTM and TSB. For immiscible liquids of high viscosity, aseptic addition of Diluting Fluid D is required to increase the flow rate. If the liquid has antimicrobial activity or contains an antimicrobial preservative, the filter is washed three times with 100 ml of the diluting fluid. Products containing lecithin, however, must use Diluting Fluid D containing the surface-active agent Polysorbate 80 to enable the dispersion of the oily substance.

Commentary: Examples of products tested by this procedure are progesterone, testosterone propionate, and dromostanolone propionate, in which the solvent is sesame oil or peanut oil.

Ointments and Oils Soluble in Isopropyl Myristate

Summary of procedure: Dissolve not less than 100 mg from each of 20 units (or 40 units if the contents are not sufficient for each medium) in 100 ml of isopropyl myristate that previously has been rendered sterile by filtration through a sterilizing membrane filter. Warm the sterile solvent, and if necessary the test material, to a maximum of 44°C just prior to use. Swirl the flask to dissolve the

ointment or oil, taking care to expose a large surface of the material to the solvent. Filter this solution promptly following dissolution, keeping the filter membranes covered with the solution throughout the filtration for maximum efficiency of the filter. Wash the membranes with two 200-ml portions of Fluid D, then wash with 100 ml of Fluid A. The media used in the test should contain 0.1% Polysorbate 80.

If the substance under test contains petrolatum, use Fluid K, moistening the membranes with about 200 ml of the fluid before beginning the filtration. Keep the membranes covered with the prepared solution throughout the filtration operation for maximum efficiency of the filter. Following filtration of the specimen, wash the membranes with three 100-ml volumes of Fluid K. Incubate the test membranes as mentioned in the previous step.

Commentary: The use of Polysorbate 80 in the final test medium is required to facilitate total dissolution of the oil or ointment being tested so that any organisms present in the material will not be isolated from the growth medium. Isopropyl myristate was found to be a satisfactory solvent for dissolving petrolatum-based ointments without adversely affecting contaminants (27). Filter-sterilized isopropyl myristate is less toxic to microorganisms than heat-sterilized isopropyl myristate (28,29). Another solvent system that has been reported to aid in the sterility testing of parenteral fat emulsions is dimethyl-sulfoxide (DMSO) (30).

Prefilled Syringes

Summary of procedure: 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 liquids miscible with aqueous vehicles.

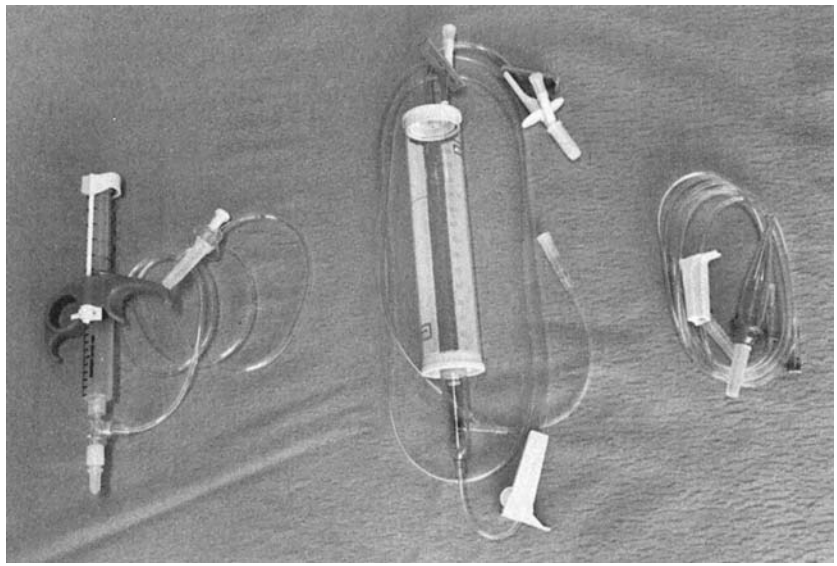

Fig. 1.6 Example of a large plastic syringe dispenser unit.

Commentary: The frequency of use of prefilled disposable syringes has increased significantly in recent years. In many instances, syringes are prefilled in hospital pharmacies. Therefore, hospital pharmacists must be trained in performing sterility tests and maintaining proper aseptic techniques in performing the tests. Aseptic technique is also especially important in cases when a needle must be attached later to the prefilled syringe.

Solids for Injection Other than Antibiotics

Summary of procedure: Constitute the test articles as directed on the label and proceed as directed for liquids miscible with aqueous vehicles or liquids immiscible with aqueous vehicles, whichever applies. Excess diluent may be added to aid in the constitution of the test article.

Commentary: Some solids for injection may not be soluble in a solvent suitable for the sterility test; consequently, the DT test must be employed.

Antibiotic Solids for Injection

Summary of procedure: For pharmacy bulk packages, less than 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, 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 liquids miscible with aqueous vehicles or liquids immiscible with aqueous vehicles, whichever applies.

For pharmacy bulk packages 5 g or larger, 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 or suspension 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 liquids miscible with aqueous vehicles or liquids immiscible with aqueous vehicles, whichever applies.

Commentary: It is important that all antimicrobial properties of the antibiotic be removed or inactivated when performing the test for sterility. Successful bacteriostasis/fungistasis validation must be performed prior to the sterility test. See Table 1.15 for a list of inactivators commonly used with antibiotics.

Antibiotic Solids, Bulks and Blends

Summary of procedure: Aseptically remove a sufficient quantity of solids from the appropriate amount of containers, 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 liquids miscible with aqueous vehicles.

Commentary: Again, it is important that all antimicrobial properties of the antibiotic be removed or inactivated when performing the test for sterility. Successful bacteriostasis/fungistasis validation must be performed prior to the sterility test. See Table 1.15 for a list of inactivators commonly used with antibiotics.

Sterile Aerosol Products

Summary of procedure: For fluid products in pressurized aerosol form, freeze the containers in an alcohol–dry ice mixture at least at −20°C for about 1 hour. If feasible, allow the propellant to escape or puncture the container before aseptically adding the contents to a sterile pooling vessel. Add 100 ml of Fluid D to the pooling vessel and mix gently. Proceed as directed for liquids miscible with aqueous vehicles or liquids immiscible with aqueous vehicles, whichever applies.

Commentary: The Millipore Corporation offers a Steritest system for use with aerosol products. This method offers many advantages over the above method, including that the Steritest system tests not only the liquid or active drug product, but also the propellant; there is no need to puncture the aerosol container, which could result in inadvertent contamination; and there is no need to freeze the container. The FDA recently proposed a rule to require all solutions for nebulization and/or inhalation to be sterile due to recalls of contaminated inhalation solutions (103).

Devices with Pathways Labeled Sterile

Summary of procedure: Aseptically pass nor 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 liquids miscible with aqueous vehicles or liquids immiscible with aqueous vehicles, 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 expel the contents into a sterile pooling vessel, then proceed as directed above.

Commentary: Many device manufacturers rely on a parametric release by which the sterility test is not performed in lieu of biological indicator data. Biological indicators are included in each sterilization load of the device to be processed and must be negative to meet the requirement of sterility.

STERILITY TESTING OF ANTIBIOTICS AND PROTEINS

Antibiotics and Antimicrobial-Containing Products

The MF method for sterility testing was developed as a solution to the antimicrobial properties of antibiotics. After filtration of the antibiotic or antimicrobial product, rinsing of the membrane is essential to remove any residual antibiotic. USP Fluid A or Fluid D are rinsing fluids of choice. For penicillins, penicillinase is added to facilitate antibiotic inactivation. The amount of penicillinase added is determined experimentally.

One important consideration in the sterility testing of antibiotics and penicillin is the issue of containment. Testing of penicillin must be in a laboratory separate from other test facilities, and cephalosporin sterility testing facilities should also be dedicated facilities.

Proteins

Gee et al. (32) described a modification of MF system for filtration of large volumes of viscous protein solutions (e.g., 25% w/v normal serum albumin). This modification operates under intermittent positive pressure through a set of membrane filter canisters.

Insulin zinc forms precipitates in sterility test media (both SCD and FTM) (6). Ascorbic acid at 1% in 0.1% peptone

(w/v) dissolves protamine zinc insulin and insulin zinc in no more than 1 minute without harming organisms.

CONTROL IN STERILITY TESTING

Sterility testing provides an estimate of the probable extent of contamination of a lot of articles. Since it is only an estimate, it must be based on sound scientific principles. Such principles primarily involve the successful incorporation of controls within each test. Sterility testing is, however, only one component of control of sterility (sterility assurance in manufacture). In the broadest sense, control starts with the environmental, personnel, and sterilization conditions implemented during the manufacture of the sterile product. Control of the quality of the environment under which the sterility test is performed is of extreme importance. The training and experience of personnel conducting the sterility test must also be controlled with regard to their understanding, use, and attitude toward strict aseptic technique. These types of controls in manufacture are discussed in a separate section. The types of control of sterility testing to be discussed in this section include the following: (a) positive control of the culture media (that is, the testing of the growth-promoting quality of each lot of media); (b) negative control of the culture media (that is, testing the sterility of the media); (c) control of the product itself (that is, obtaining knowledge about the bacteriostatic and/or fungistatic activity of the product prior to its being subjected to a sterility test); and (d) specific controls when using the MF technique.

Positive Controls

The absence of growth in sterility test samples at the completion of the test indicates that the product is sterile insofar as assumptions and limitations of the test are considered, that is, it meets the requirements of the test. However, this conclusion can be made only with the assurance that growth would have occurred during the sterility test period had microorgan-

isms actually been present. The USP and EP growth promotion tests are designed to serve as a positive control for each lot of sterility test media. Each lot is inoculated with 10 to 100 of the microorganisms listed in Tables 1.13 and 1.14. Growth of these microorganisms must occur in the appropriate medium within 7 days of incubation. The evidence of growth in duplicate test containers compared with the same lot of medium containing no microbial inoculum qualifies the test medium to be used for sterility test purposes. The USP allows for the growth promotion test to be the positive control run simultaneously with the actual sterility test with the understanding that the test becomes invalid if the medium does not support the growth of the inoculated microorganisms. However, if tested media are stored, additional tests are prescribed for particular storage conditions.

Negative Controls

Negative controls consist of containers of culture media without addition of product sample or microbial challenge. The purpose of negative control samples is to verify the sterility of the medium before, during, and after the incubation period of the sterility test. If microbial growth is detected with a negative control, the medium was not sterilized properly, contamination was introduced accidentally during the test procedure, or there exists an inefficiency in the container or packaging system. If such microbial growth in a negative control occurs and in the absence of evidence from the environmental monitor, equipment, or personnel of accidental contamination, it becomes a clear indication for retesting the product.

Bacteriostatic and Fungistatic Testing

If a sterility test is negative (no growth), there must be the assurance that growth was not inhibited by the antimicrobial properties of the product itself. The USP provides a procedure for determining the level of bacteriostatic and fungistatic activity of a product or material prior to its being tested for ste-

Table 1.13 Test Microorganisms Required by the USP for Use in the Growth Promotion and Bacteriostasis/Fungistasis Test Used in Sterility Testing[a]

Medium	Test microorganism[b]	Incubation temperature (°C)	Condition
Fluid thioglycollate	*Staphylococcus aureus* (ATCC 6538)[c]	32.5 ± 2.5	Aerobic
	Pseudomonas aeruginosa (ATCC 9027)[d]	32.5 ± 2.5	Aerobic
	Clostridium sporogenes (ATCC 11437)[e]	32.5 ± 2.5	Aerobic
Alternative thioglycollate[f]	*Clostridium sporogenes* (ATCC 11437)	32.5 ± 2.5	Anaerobic
Soybean-casein digest	*Bacillus subtilis* (ATCC 6633)	22.5 ± 2.5	Aerobic
	Candida albicans (ATCC 10231)	22.5 ± 2.5	Aerobic
	Aspergillus niger (ATCC 16404)	22.5 ± 2.5	Aerobic

All organisms are required to show visible growth within not more than 7 days of the original test.

[a] ATCC cultures represent reference species, and their use for compendial test is predicated on them not being subjected to procedures that may alter their properties. Such procedures include indefinite numbers of subcultures with no standardization of conditions. For this reason, the USP has proposed that seed lot culture techniques may be used and that the viable microorganisms used be no more than five passages removed from the reference species.

[b] Available from the American Type Culture Collection, 10801 University Boulevard, Manassas, VA 20110-2209.

[c] An alternative to *Staphylococcus aureus* is *Bacillus subtilis* (ATCC 6633).

[d] An alternative microorganism is *Micrococcus luteus* (ATCC 9341).

[e] An alternative to *Clostridium sporogenes*, when a nonspore-forming microorganism is desired, is *Bacteroides vulgatus* (ATCC 8482).

[f] Used for sterility test of devices that have tubes with small lumens.

Table 1.14 Test Microorganisms Required by the EP for Use in the Growth Promotion and Bacteriostasis/Fungistasis Test Used in Sterility Testing[a]

Medium	Test microorganism[b]	Incubation temperature (°C)	Condition
Fluid thioglycollate	*Staphylococcus aureus* (ATCC 6538)[c]	32.5 ± 2.5	Aerobic
	Pseudomonas aeruginosa (ATCC 9027)[d]	32.5 ± 2.5	Aerobic
	Clostridium sporogenes (ATCC 19404)[e]	32.5 ± 2.5	Aerobic
Soybean-casein digest	*Bacillus subtilis* (ATCC 6633)	32.5 ± 2.5	Aerobic
	Candida albicans (ATCC 10231)	22.5 ± 2.5	Aerobic
	Aspergillus niger (ATCC 16404)	22.5 ± 2.5	Aerobic

Note that there is no alternative fluid thioglycollate medium as in USP XXV.

[a] ATCC cultures represent reference species, and their use for compendial test is predicated on them not being subjected to procedures that may alter their properties. Such procedures include indefinite numbers of subcultures with no standardization of conditions. For this reason, the USP has proposed that seed lot culture techniques may be used and that the viable microorganisms used be no more than five passages removed from the reference species.

[b] Available from the American Type Culture Collection, 10801 University Boulevard, Manassas, VA 20110-2209.

[c] An alternative to *Staphylococcus aureus* is *Bacillus subtilis* (ATCC 6633).

[d] An alternative microorganism is *Micrococcus luteus* (ATCC 9341).

[e] All bacteria are required to demonstrate visible turbidity within 3 days of incubation, and all fungi are required to demonstrate visible turbidity within 5 days of incubation.

rility by the DT or MF test. Basically, the procedure calls for adding product to containers of culture media in volumes corresponding to those that would be used for testing the product containing 10 to 100 of the microorganisms listed in Table 1.13 and comparing with medium-inoculum controls without the product. If the material possesses bacteriostatic or fungistatic activity, then the product-media will show decreased or no microbial activity compared to control culture media. If this is the case, then procedures must take place for the proper inactivation of these bacteriostatic/fungistatic properties. Either a suitable sterile inactivating agent must be found or the material and medium must be adequately diluted to overcome the static effects. If at all possible, the MF test should be applied for those materials found to be bacteriostatic or fungistatic. When MF is used, similar comparisons are made of incubated filters through which product and suitable diluting fluid have been passed, each containing the same added microorganisms.

Specific inactivating or diluting methods used for a few drugs or drug products known to be bacteriostatic or fungistatic are listed in Table 1.15.

Controls for Membrane Filtration Techniques

The MF test relies on the ability to produce sterile equipment and to have aseptic conditions under which to conduct the test. Three basic control procedures are recommended in separate experiments:

1. The membrane filters are challenged after their sterilization cycle for their ability to retain microorganisms.
2. The exposure times for agar settling plates used to monitor the environment are validated.
3. The cleaning procedures used to remove bacteriostatic and/or bactericidal residues from equipment following the MF test must be validated. This is especially important for the equipment involved in the sterility testing of antibiotics.

Table 1.15 Inactivation of Bacteriostatic/Fungistatic Agents in Sterile Products Tested by the Direct Transfer Sterility Test

Agent	Method of inactivation
Mercurials	
Phenylmercuric nitrate (1:50,000 conc.)	10 ml FTM
Merthiolate (1:10,000 conc.)	10 ml FTM or 12% sodium thiosulfate
Phenol	Adsorb on 0.1% Darco or 0.03% ferric chloride or dilute 0.5% phenol in 50 ml culture medium
Benzalkonium chloride	Lecithin and polysorbate 80
Sulfonamides	p-Aminobenzoic acid
Penicillin	Penicillinase
Cephalosporins	Cephalosporinase
Streptomycin	Cysteine HCl 2% in acid medium
Cresol	Dilute 0.35% in 60 ml culture medium
Chlorobutanol	Dilute 0.5% in 40 ml culture medium
Barbiturates	Dilute to 0.2% in culture medium with a pH of about 7.0
Aminoglycosides	Acetyl-coenzyme A[a]

[a] A. S. Breeze and A. M. Simpson, An improved method using acetyl-coenzyme A regeneration for the enzymatic inactivation of aminoglycosides prior to sterility testing, *J. Appl. Bacteriol*., *53*, 277 (1982).

VALIDATION OF THE STERILITY TEST

For every product that is tested for sterility, the sterility test method must be validated for that product. What this means, simply, is that prospective validation studies must be performed to collect data to prove that the sterility test can detect microbiological contamination in the product. Validation of the sterility test for a particular product involves adding small but known concentrations (<100 CFU) of various microorganisms to the final rinse and then demonstrating recovery of the

organisms using the sterility test methodology. Table 1.13 provides the test organisms required by USP XXV. Table 1.14 provides the test organisms required by the EP. While the EP and USP chapters on sterility testing are now considered to be "harmonized," if the practitioner desires to test a product for sterility release and that product is required to meet EP and USP requirements, there are several key points to consider.

1. Organisms from tables 1.13 and 1.14 will have to be used in the bacteriostasis and fungistasis test, and *Bacillus subtilis* must be tested in both FTM and TSB. Upon incubation of the challenge containers, all bacteria must show visible growth within 3 days of the test, and all fungi must show visible growth within 5 days of the test.
2. Even if the product is terminally sterilized, the final sterility test must incubate for 14 days (if the MF technique is used) to satisfy the EP requirement.
3. ATCC 19404 must be used for the *Clostridium sporogenes* challenge to satisfy the EP requirement, while ATCC 11437 must be used for the *C. sporogenes* to satisfy the USP requirement when performing the bacteriostasis and fungistasis test.
4. When the DT test is employed, the initial transfer test is required to incubate for 14 days for EP, but only 7 days for the USP test.

LIMITATIONS OF THE USP/NF REFEREE STERILITY TEST

The USP referee sterility test suffers from at least three limitations: (a) the invariant uncertainty that the small sample used in the test reliably represents the whole lot, (b) the inability of the culture media and incubation conditions to promote the growth of any and all potential microbial contaminants, and (c) the unavoidable problem of occasional accidental contamination of the sterility test samples. Ernst et al. (33) believe that impeccable control of three phases of the steriliza-

tion and sterility testing of parenteral products will alleviate many of the problems of sterility testing: (a) knowledge and understanding of the sterilization process, (b) avoidance of unfavorable environmental conditions during manufacture and testing, and (c) education of personnel in the procedures of sterility testing.

The Problem of Sampling and Statistical Representation

The probability of accepting lots having a given percentage contamination is related to the sterility test sample size rather than to batch size (34). For example, if a batch is 0.1% contaminated (1 nonsterile unit in 1000 units) and 10 units are sampled for a sterility test, the probability of finding 1 of those 10 samples to be the 1 contaminated unit in 1000 is not significantly different if the batch size were 1000, 2000, or 5000). Increasing the sample size from 10 to 20 to 50 units per batch, however, affects the probability of accepting the batch as sterile to a more significant degree than does the increase in batch size, assuming that the increase in batch size does not increase the level of contamination. This phenomenon is depicted in Table 1.16. The probability rate does not change as the batch size is increased, but does change as the sample size is increased. Of course, a key factor is that the contamination rate remains at 0.1% as the batch size increases. This, in reality, may not be true, especially for aseptically filled products. Hence, as the contamination rate increases with batch size, the probability of acceptance decreases for the same sample size.

Table 1.16 Probability of Accepting a Batch as Sterile Assuming the Contamination Rate to be Constant at 0.1%

Sterility test sample size	Batch size		
	1000	2000	5000
10	0.99	0.99	0.99
20	0.98	0.98	0.98
50	0.95	0.95	0.95

The relationship of probability of accepting loss of varying degrees of contamination to sample size is given in Table 1.17 (35). Three details may be learned assuming the data in Table 1.17 are real:

1. As the sample size is increased, the probability of accepting the lot as sterile is decreased.
2. At low levels of contamination, (e.g., 0.1%), the odds of ever finding that 1 contaminated sample in 1000 units are so small that one must face the fact that lots are going to be passed as sterile but somewhere, at some time, some patient is going to receive that nonsterile sample (even at a contamination rate of 1% with 20 sterility test samples, it must be realized that such a lot will be passed as sterile 82% of the time).
3. Realistically, a batch must be grossly contaminated for the sterility test to detect it. This fact was concluded at a 1963 conference on sterility testing in London (36), at which experts in sterility testing recognized that the lowest contamination rates that can be detected with 95% confidence are 28% with a sample size of 10, 15% with a sample size of 20, and 7% with a sample size of 40 units.

A sample size of 20 units is shown in Table 1.18. As an example, if it is assumed that only 1 unit in a batch of 100,000 units is contaminated (0.001%), the probability that the 1 contaminated unit is among the 20 sterility test samples taken at random is 0.0002, or 2 times in 1 million sterility tests. Table 1.19 presents an example of why dependence on sampling and sterility is, in fact, a futile attempt to prove the sterility of a lot.

If there is contamination, an acceptable level of acceptance is 1 in 100 lots tested, the probability that 0.1% of the lot is contaminated is achieved with 40 samples, but with lower contamination levels, many more samples would be required. For example, sample requirements for 0.01% contamination would be 450 samples; for 0.001% contamination, it is 4500 samples; and for 0.0001% contamination, it is 45,000 samples.

Table 1.17 Relationship of Probabilities of Accepting Lots of Varying Assumed Degrees of Contamination to Sample Size

Number of samples tested (n)	Probability of accepting the lot ("true" percentage contamination of lot)					
	0.1	1	5	10	15	20
10	0.99	0.91	0.60	0.35	0.20	0.11
20	0.98	0.82	0.36	0.12	0.04	0.01
30	0.95	0.61	0.08	0.01		
100	0.91	0.37	0.01			
300	0.74	0.05				
500	0.61	0.01				

Table 1.18 Probability of Finding at Least One Nonsterile Unit in a Sample Size of 20 Subjected to a Sterility Test

Assumed percentage nonsterile units in the lot	Probability of finding at least 1 nonsterile unit
0.10	0.01980
0.05	0.00995
0.02	0.00399
0.01	0.00199
0.005	0.00100
0.002	0.00040
0.001	0.00020

This illustrates the futility of attempting to determine sterility levels (when low) by sterility tests alone.

Take the following example: If sterility tests have been done, using one medium and 20 samples on each occasion, and only 2 inoculated tubes showed growth, the proportion contaminated may be 2/200. However, if when a positive result

Table 1.19 Futility of Depending on Sampling and Sterility Tests for Sterility Assurance of a Lot

	Probability of finding all negatives in samples of different sizes for various levels of contamination		
n	$p = .1$	$p = .01$	$p = .001$
10	0.35	0.90	0.99
20	0.12	0.82	0.98
40	0.01	0.69	0.96
160		0.20	0.85
640			0.53

If the proportion of contaminated units in the lot is p, then the proportion of noncontaminated units in that lot is $1 - p$. Let that be designated q. Then, the probability of finding noncontamination (i.e., acceptance) of that lot with taking n samples for testing is $(q)^n$.

was obtained, the tests were repeated with another 20 samples each time, with negative results, the proportion contaminated may be 2/240, that is, 0.0083, and the proportion not contaminated 0.9917. If that lot were contaminated to the determined level, to reduce the probability of acceptance to 1 in 100 would require about 550 samples to be taken. Not only is such a number not feasible, the probability of adventitious contamination in sterility tests (ranging from 0.2% to 3% of tests, see the section "Problem of Accidental Contamination") makes even that possibility likely to yield an unreliable result.

A mathematical equation for calculating the probability P of releasing lots at different levels of contamination was developed by Armitage (37):

$$P = e^{-mv}$$

where m is the number of microorganisms per milliliter, and v is the volume in milliliters of the test sample. For example, if 10 microorganisms are present per 100 ml and the test sample is 100 ml (20 containers $\times$ 5 ml per container), the probability of releasing the lot of this contaminated product is 0.0000454. Like the presented data in the preceding tables, this equation shows that relatively small sample sizes and/or low contamination levels result in lots being judged to meet the sterility test requirements when, in fact, a finite number of articles in the lot are nonsterile. Thus, claims for low probability levels of nonsterility cannot realistically be proven by the random sampling procedure of the USP sterility test, and sterility assurance must be achieved by appropriate control measures in manufacture (see the section, "Support Techniques and Procedures for Sterility Assurance"). In fact, with low levels of nonsterile units in a lot, any reasonable sampling plan would not provide realistic results (see Table 1.19). This does not even consider the finite probability of inadvertent contamination entering the product during the sterility test procedures.

Problem of Supporting the Growth of Microbial Contaminants

No single medium will support the growth of all microbial forms (that is, bacteria, molds, fungi, and yeasts). FTM will not recover very low levels of some aerobic spore formers such as *Bacillus subtilis* (14). Friedl (38) reported that TSB gave more efficient recovery of small numbers of *B. subtilis* and *C. sporogenes* spores than in FTM. TSB, being strictly an aerobic medium, will not support the growth of the genus *Clostridia*. On the other hand, while FTM effectively supports the growth of various strains of *Clostridia*, it has been reported that sodium thioglycollate is toxic to *Clostridia*, and this antioxidant should be replaced by cysteine hydrochloride (39).

TSB is incubated at 20–25°C to permit adequate growth of facultative organisms such as enterobacteria (*Escherichia coli, Salmonella, Shigella, Proteus, Serratia marcescens,* and *Flavobacterium*) and many yeasts. FTM is incubated at 30–35°C to detect mesophilic bacteria. These sterility media, therefore, are not incubated at temperatures conducive to the growth of psychophiles (predominantly pseudomads) and thermophiles (predominantly bacilli). According to Bruch (40), TSB and FTM do not contain the necessary nutritional ingredients to support the growth of obligate halophiles, osmophiles, or autotrophs.

Problem of Accidental Contamination

Growth that occurs in sterility test media must be ascertained to have originated from the test sample and not from the culture media or from an external source during the execution of the test. Such a determination can be made only to a limited extent. The use of negative controls eliminates one source of contamination, that resulting from nonsterile culture media. Thus, a positive sterility test result is concluded to be true (the test sample is contaminated) unless it can be shown to be false (contamination was accidently introduced during the test procedure). The problem of false positives is widespread and cannot be completely eliminated.

The percentage of false-positive sterility tests is reported in the range from 0.2% to 3% (1,2,41). In a poll conducted by one of us, of 10 pharmaceutical companies involved in sterile product sterility testing, the range of inadvertent contamination found during sterility testing was 0.1% to 5%. The most common types of microbial contaminants found in false-positive sterility test samples are listed in Table 1.20.

False-positive sterility tests result also from contaminants located in the environment (air and surfaces), on people conducting the test (hands, breath, hair, clothing, etc.), or on the equipment used in conducting the test (nonsterile membrane filler assemblies, scissors, forceps, filters, etc.). Contamination being accidently introduced by the environment can be reduced significantly by performing a monitored environmental sterility test in a laminar air flow (LAF) workbench (see the section, "Laminar Air Flow"). For example, Parisi and

Table 1.20 Examples of Microorganisms Found in Aseptically Filled Parenteral Products (Found in Antibiotic and Nonantibiotic Products)

Microorganism type	Source	Examples
Gram positive cocci	Human contamination	*Staphylococcus* *Micrococcus* *Streptococcus*
Gram negative bacilli	Water	*Pseudomonas*
Coliforms (also GMB)	Fecal contamination	*Escherichia* *Enterobacter* *Citrobacter*
Gram positive bacilli	Dirt, dust	*Bacillus* *Clostridium* *Corynebacterium*
Gram negative cocci	Rare pathogens	*Neisseria*
Molds	Air, dust	*Penicillium* *Aspergillus*
Yeast	Air, dirt	*Candida* *Rhodotorula* *Saccharomyces*

Borick (42) found that the percentage of false positives during sterility testing fell from 1.61% when done in conventional sterile rooms to 0.63% when done in an LAF workbench. These same authors also reported that 2361 colonies were recovered on 765 agar settling plates located in the conventional sterile room, while only 75 colonies were recovered on 299 agar setting plates located on an LAF workbench. Thus, while LAF workbenches do not completely eliminate the incidence of contamination, they do significantly reduce the potential problem provided the results from the settling plates are used to indicate corrective actions.

The single largest contributor of accidental contamination in sterility test samples is the person or people performing the test. Personnel-induced accidental contamination primarily results from a lack of strict adherence to good aseptic technique. Good aseptic technique involves many considerations, including apparel, eye-hand coordination, concentration, and the desire to be as careful as possible. An excellent resource for training on aseptic techniques is the chapter by Luna (43).

Accidental or adventitious contamination is one of the greatest problems interfering in the interpretation of sterility test results in hospital pharmacies (44). Bernick et al. (45) suggested that contaminated intravenous admixtures are not contaminated during the admixture process, but rather are contaminated from microorganisms introduced during the sterility testing procedure. Such admixture processing should be carried out in appropriate hospital pharmacy facilities and not in the patient care areas. Sterility testing should be an essential component in the monitoring of intravenous solutions and admixtures in hospital pharmacy practice (46–51).* Several

* This has been more recently substantiated by the Draft Guidelines on Quality Assurance for Pharmacy-Prepared Sterile Products (*Am. J. Hosp. Pharm.*, *49*, 407–417, 1992), which propose that pharmacists establish quality assurance procedures, including sterility tests, for pharmacies that are involved in the preparation of sterile products.

methods have been suggested for evaluating sterility of intravenous admixtures (52–56). However, the problem of adventitious contamination and the limitations resulting from this problem that affect the interpretation of the sterility test must be recognized. The National Coordinating Committee for Large Volume Parenterals (NCCLVP) strongly recommended that suitable education programs in hospitals and colleges be developed to educate and train personnel involved in the preparation and administration of sterile medication (49). NCCLVP also recommends developing procedures for in-use testing of LVPs suspected of contamination.

ISOLATION CHAMBERS AND ROBOTIC STERILITY TEST UNITS

As previously discussed, false-positive sterility tests occur because of inadvertent contamination of the sample in the sterility test laboratory. Such contaminations are of a finite probability as long as human manipulation is involved. Concerns over such unreliabilities of the sterility test have given rise to new technologies designed to remove as much as possible the human element involved in sterility testing.

d'Arbeloff et al. (57) have described four robotic sterility test systems: (a) Hoffmann–La Roche first documented robotic system in the pharmaceutical industry (58); (b) Farmitalia; (c) Takeda; and (d) Precision Robots, Incorporated, Autotest 1000. Undoubtedly, there are other commercially available or company-built systems being used today. Each robotic system is designed to eliminate the tasks that contribute to or cause the inadvertent contamination problems causing false-positive sterility tests. Each of the robotic systems described by d'Arbeloff et al. (57) was successful in eliminating adventitious contamination. For example, the Roche system had not had a confirmed false-positive test in over 4 years of operation.

Major disadvantages of the robotic sterility test systems are their expense of installation and maintenance, slower

speed in conducting sterility tests, and increased complexity of setting up, using, and maintaining the system.

Validation of robotic sterility test systems involves at least five elements:

1. Validating the current manual procedure, which should be a procedure used over many years and having a known and low rate of adventitious contamination.
2. Validating all the robotic operations, both hardware and software.
3. Validating the laminar air flow patterns in the system.
4. Validating the particle levels in and around the test areas.
5. Validating the disinfection of the system. Peracetic acid should not be used as a disinfectant in robotic systems (it can be used in isolation chambers) because of it causes corrosive problems. Acceptable disinfectants for robotic systems include Ampfil, Sporicidin, or 3% hydrogen peroxide.

Finally, d'Arbeloff et al. (57) recommend that the robotic system be challenged by introducing contamination, (e.g., worst-case operator contamination in introducing vials into the system) to show that the design of the system, its optimized laminar air flow, and the removal of any further human intervention indeed remove seeded microbial contamination on product containers.

While the LaCalhene isolation chamber was the most widely used sterility test chamber system in the parenteral industry in the 1980s to mid-1990s, the use of hard-walled isolators has become the most recent trend in sterility testing (see Fig. 1.7) (102). The LaCalhene module (Figure 1.8) is made of polyvinyl chloride supported externally by a framework of stainless steel rods. The barriers can be accessed by the operator through either glove sleeves or half-suits. Materials can be introduced into or removed from these barriers through a double door transport port sterile transfer door. Room air enters and exits through a 0.3-μm high-efficiency

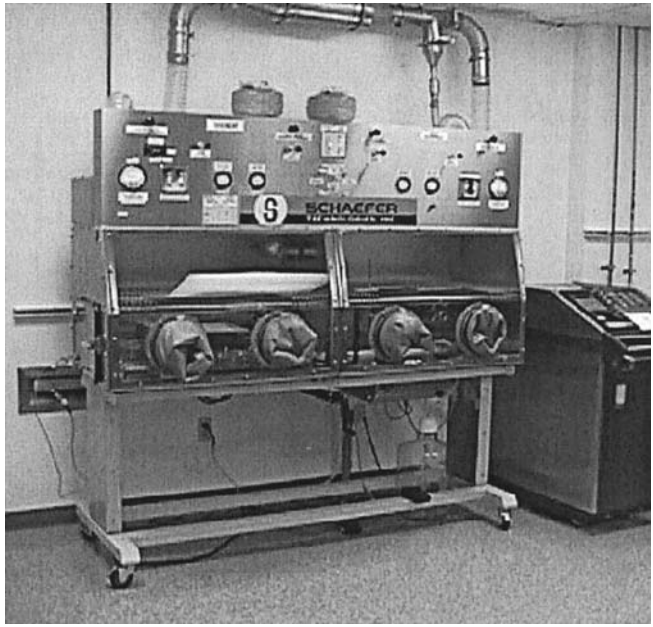

Fig. 1.7 Example of a Schaeffer Engineering hard-walled isolation barrier system for sterility testing (courtesy of Baxter Pharmaceutical Solutions LLC, Bloomington, Indiana).

particulate air (HEPA) filter. LaCalhene offers many different types of isolation chambers in terms of design and function. All sterility test operations—product container surface decontamination, sterility test manipulations, and incubation of samples—occur within the barrier system.

One of the major aspects of the isolation chamber is the sterilization and its validation of all surfaces within the chamber and product containers and other items brought into the chamber. The original method of surface sterilization was the use of peracetic acid as a spray. Davenport (59) described the use of peracetic acid as a sterilizer in LaCalhene sterility testing chambers and the methods used to validate that the sterilant is effective in destroying the biological indicator (*Bacillus circulans UC9951*).

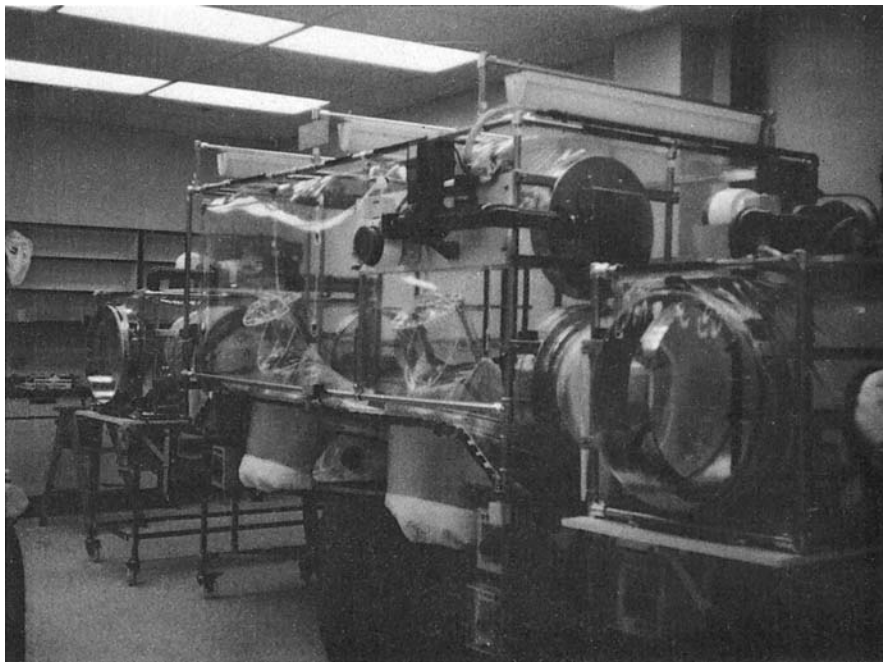

Fig. 1.8 Example of a LaCalhene isolation barrier system for sterility testing (courtesy of Eli Lilly and Co., Indianapolis, Indiana).

The most commonly used method of surface sterilization today is VPHP (vapor phase hydrogen peroxide) (102). The VHP 1000® manufactured by the Steris Corporation (formerly AMSCO) (see Fig. 1.9) is widely used in the pharmaceutical industry in conjunction with barrier isolation systems. VPHP is less corrosive to metals such as stainless steel than is peroxyacetic acid.

The advent of isolation chambers and robotic sterility test systems has challenged the long-held level of acceptability of false positives. The current level of 0.5% rate of false positives, both in aseptic manufacture and sterility testing, may no longer be acceptable as it is becoming more plausible to have a much lower rate of contamination. Cooper (60) points out that the industry may be facing a two-tier situation in which

Fig. 1.9 The VHP 1000® unit used for surface sterilization within isolators (courtesy of Steris, Erie, Pennsylvania).

companies that have implemented isolation chambers or sterility test robotics will be expected to maintain lower levels of false positives than companies that still perform sterility tests under the conventional laminar flow laboratory method. It will be very interesting to see how this situation will be addressed by the FDA and other regulatory bodies in the years ahead.

Validation of Barrier Isolation and Associated Sterilization Systems

Like any other process in the pharmaceutical industry, barrier isolation used for sterility testing must be shown to reproducibly deliver the desired result. Because of their complexity, there are several parameters to consider in the design and validation of isolation systems. USP chapter <1208> provides guidance for the design and validation of isolator systems for use in sterility testing. The guidelines in USP <1208> are

summarized below, as are common practices in the validation of sterility testing isolators. For a complete description of <1208>, consult the most current USP.

The steps and considerations that are essential to the isolator validation and design are outlined below:

Design

By design, an isolator which is used for sterility testing should be equipped with filters capable of microbial retention. HEPA filters are required, but ULPA (ultra-low penetration air) filters may be substituted. While the isolator is at rest, it must meet the particulate requirement for a class 100 area as described in U.S. Federal Standard 209E (71). There is no particulate requirement while the unit is in operation during a sterility test, and there is no requirement for air velocity or air exchange rate. The isolator should be leak-proof, but it may exchange air with the surrounding environment. While direct openings with to the surrounding environment should be avoided, air overpressure can be employed to maintain sterile conditions within the isolator. Air overpressure should also be employed to help avoid ingress of nonsterile air in the event of an unexpected leak.

Location of the Isolator

The isolator does not need to be installed in a classified clean room, but the surrounding room should be limited to essential staff. Environmental monitoring of the surrounding room is not required.

The surrounding room should have sufficient temperature and humidity control to maintain operator safety and comfort, to allow for proper operation of the associated sterilizer (the air should exhaust to an outside source for safety reasons) unit, and to allow for proper operation of the isolator. The temperature within the room should be as uniform as possible to avoid the formation of condensation within the isolator.

Installation Qualification

The installation qualification (IQ) should include a detailed description of all of the mechanical aspects of the system, such as dimensions, internal configuration, serial numbers of the equipment, blueprints, purchase orders, electrical supply, specifications, exhaust, vacuum supply, and equipment manuals. All documentation should be reviewed for accuracy. The following pieces of documentation are recommended:

Equipment: The equipment is listed with critical design specifications. The IQ should verify that the appropriate design specification was received and that all equipment was installed per the manufacturer's requirements.

Construction materials: The critical components of the system are checked for compliance with the design specification and for compatibility with the method of sterilization.

Instruments: System instruments are listed with their calibration records.

Utility specifications: All utilities that are required for operation as defined in the operating manuals and diagrams are verified. Any connections between electrical and/or exhaust systems are inspected and verified to conform to specifications.

Filter certification: HEPA filters are tested and certified, and copies of the certifications are included.

Computer software: All computer software is listed with name, size, and version number. Any master copies should be properly labeled and stored should the need for a backup arise.

Operational Qualification

The operational qualification (OQ) step verifies that the isolator system operates within conformance to functional aspects.

Operational performance check: All alerts and alarms should be tripped and proper functioning verified.

Isolator integrity check: The integrity of the isolator should be

verified to be free from leaks. The leak test is important to preclude contamination and for operator safety. The overpressure set point should be established and shown to be maintained during operation.

Sterilization cycle verification: Verification of relevant temperature and/or humidity control during the sterilization cycle should be demonstrated. Humidity may be especially important depending on the type of sterilizing gas that is used. The concentration and distribution of the sterilizing gas should be measured using chemical indicators. After sterilization, verification that the sterilizing gas has been removed (by aeration) to an acceptable level should be demonstrated by quantitative methods.

Sterilization cycle development: On completion of the OQ, cycle development parameters are established to achieve sterilization of the isolator unit. Sterilization should be demonstrated by use of the bioburden approach, through the use of biological indicators (BIs) of a known concentration, or by the half-cycle approach.

Note that *Bacillus stearothermophilus* is an appropriate choice as a BI in this application, as it is more resistant to VPHP than most environmental isolates (104).

Performance Qualification

The performance qualification (PQ) verifies that the systems are functioning in compliance within its operational requirements. On completion of the PQ phase, sterilization efficacy should be established. For sterilization validation, the interior surfaces of the isolator, articles within the isolator, equipment in the isolator, and sterility test articles should be rendered sterile after processing. BI kills of 10^3 to 10^6 are commonly used in the pharmaceutical industry in association with sterility testing isolators.

Since most pharmaceutical companies produce many different package presentations, the minimum load (an empty chamber) and maximum load (the maximum number of test

articles) approach is helpful when performing the PQ of the isolator.

False-Negative Evaluation

Because certain materials are adversely affected or absorb sterilizing agents, it is important to demonstrate that the method of sterilization would not destroy any microbial life since the point of a sterility test is to detect low levels of organism in the finished product.

Various containers, media, and rinsing fluids should be inoculated with low levels of organisms (<100 CFU) and subjected to the sterilization cycle (suggested organisms are listed in Table 1.13). After sterilization, the level of inoculum should be verified to prove that organisms are not destroyed during the cycle. In the event that organisms are adversely affected, a new container closure should be selected before use with the isolator system.

SUPPORT TECHNIQUES AND PROCEDURES FOR STERILITY ASSURANCE

Because sterility assurance is based on a probability function, sterility can never be proven unless the entire contents of a lot are subjected to a sterility test. Even this is not theoretically possible because of the need to use at least two different media for the test. In addition, as already discussed, the sterility test itself has certain limitations. Therefore, product sterility cannot be tested with absolute assurance that every container of sterile product is sterile. However, assurance of product sterility can be achieved with a high degree of probability by the employment of and adherence to various procedures, of which sterility testing is only an adjunct. These include (a) sterilizer and sterilization method validation using physical and biological indicators; (b) impeccable control of the environmental conditions under which the parenteral product is manufactured, particularly when aseptic processing is performed; and (c) thorough training of personnel of the strict aseptic techniques

required for performing the sterility test. Any sterility test should be done in an environment equal to or more controlled than that used for aseptic processing. An important part of long-range sterility assurance is adequate documentation of the validation, monitoring, personnel training/use of aseptic technique, and batch manufacturing procedures used.

Sterilizer and Sterilization Method Validation

The assurance of parenteral product sterility primarily depends on the process used to sterilize the product. The greater the control of the process, the greater the assurance of sterility. Sterilization process control involves knowledge and management of *process* variables such as temperature, pressure, concentration, humidity, load configuration, and filter integrity and of *product* variables such as solution composition and viscosity, packaging specifications, and microbial content.

Four basic methods are employed to sterilize parenteral products.

1. Heat, both wet (steam) and dry
2. Gas, primarily ethylene oxide
3. Radiation, primarily cobalt 60, gamma irradiation, and electron beam
4. Filtration through bacterial retentive membrane fibers

The mechanics and engineering of each of these processes must be understood and properly controlled for the process to provide additional assurance of product sterility.

Simmons (61) has elaborated on the engineering aspects of validating steam, dry heat, and ethylene oxide sterilizers. Filter integrity testing has been adequately described by Reti and Leahy (62).

Once the sterilizing system itself has been qualified (i.e., for capability to achieve sterilization), then the process of sterilization can be validated. Validation of the process involves both physical and biological methodology. Physical methods include temperature measurement, gas concentration or irradiation dose monitoring, and the use of mathematical expres-

sions such as the F value equation (63). Biological methods involve the employment of biological indicators to evaluate the ability of the sterilization process to destroy or eliminate an inordinately high concentration of known resistant microorganisms under conditions identical to those found in the sterilization of the actual parenteral product. They also are used to monitor a validated sterilization cycle.

Biological Indicators

Greater confidence in sterility assurance has arisen because of the increased acceptance and employment of BIs during the development of the sterilization cycle or system (64). If the sterilization process is shown with a high degree of probability to destroy, say, 10^6 spores of known resistance to the process, then a batch of parenteral product exposed to that same process will result in a sterile product. This may be roughly confirmed by the sterility test. BIs are microorganisms, usually spore forms, known to be as resistant to destruction by a given sterilization process as any microbial form known. BIs are used to verify the effectiveness of a sterilization process because if the process can destroy the BI of known concentration, it is assumed that the process will also destroy all other microbial contaminants potentially present in the product. Of course, this assumption is controversial, and many experts question how far one can really depend upon it.

Microorganisms recognized by the USP as BIs for the various sterilization processes are given in Table 1.21. However, one is not restricted from employing other types of microorganisms as BIs if they better serve the needs of the particular process. Several species of *Bacillus* spore are known to be more resistant than the strain of *Bacillus subtilis* var. *niger* (ATCC 9372), the USP biological indicator for monitoring ethylene oxide sterilization (66). *B. pumilus* (ATCC 27142) has demonstrated the same degree of resistance to ethylene oxide as *B. subtilis* var. *niger* (66).

Vegetative cells, rather than bacterial spores, are employed in testing and validating filtration sensitization. *Bre-*

Table 1.21 Performance Characteristics of Biological Indicators on Paper Strips

Culture	Sterilization process	D value	Approximate spore content	Survival time (not less than)	Kill time (not more than)
Bacillus stearothermophilus spores (ATCC 7953 or 12980)	Saturated steam at 121 ± 0.5°C VPHP	1.3–1.9 min	10^6	3.9 min	19 min
Bacillus subtilis var. niger (ATCC 9372)	Ethylene oxide at 54 ± 2° and relative humidity 60 ± 10%; 600 ± 30 mg/liter	2.6–5.8 min	10^6	7.8 min	58 min
	Dry heat at 160 ± 5°C	1.3–1.9 min	10^6	3.9 min	19 min
Bacillus pumilus (ATCC 27142)	Ionizing radiation				
	Wet preparations	0.16–0.24 Mrad	10^6	0.6 Mrad	2 Mrad
	Dry preparations	0.12–0.18 Mrad	10^6	0.45 Mrad	1.5 Mrad
General requirement		D ± 20%	10^6	3 D	10 D

vundimonas diminuta (ATCC 19146), a vegetative organism selected for its small size (approximately 0.3 μm), is the organism of choice for evaluating the retention ability of 0.2-μm sterilizing membrane filters. Recent controversy has arisen over the ability of a 0.2-μm membrane to retain all organisms; therefore, the use of 0.1-μm filters or redundant 0.2-μm filtration has become more common in the pharmaceutical industry.

The USP provides a general description of BIs, which are available either as liquid suspensions or as dried preparations on carriers such as paper strips, glass, plastic beads, ampules, or stainless steel strips. BIs used as a spore suspension should be added to representative units or to units similar to those of the lot to be sterilized. The BI must demonstrate a challenge of the sterilization process that exceeds the challenge of the natural bioburden. BIs must be properly standardized so that the BI units in the lot all exhibit the same degree of resistance to the sterilization process when used in the same manner, even if varying at different times within the dating period of the BI. The BI inoculum must be prepared under the supervision of trained microbiologists in order to maintain and standardize BI cultures of known purity, identity, and resistance. Every commercially prepared BI product must be labeled according to the relevant USP general notices on labeling, as well as with its spore content and performance characteristics, such as decimal reduction time (D value) under given sterilization parameters, directions for use, and recommendations for disposal.

Several interesting review articles have been written on the principles and applications of BIs. Borick and Borick (3) were the first to write a lengthy discussion on the use of BIs versus the use of regular sterility test samples. Bruch (40) presented a strong case for using BIs as a means of evaluating the probability of sterility of products sterilized by methods other than saturated steam under pressure. Myers and Chrai (67) reviewed the biology of microbial resistance and application of bioindicators in designing and monitoring sterilization cycles.

Caputo and Mascoli (68) suggested a four-step process in the design of a BI system for validating the efficacy of a sterilization cycle. In the first step, the microorganism to be used as the BI is selected and propagation procedures are developed to ensure the consistent production of a homogeneous population of BI with the desired resistance to the sterilization process. Second, the D value (the time required to reduce the microbial population by 90% or through one log cycle) is determined for the selected BI. Factors that must be considered and that affect the D value of a particular BI were discussed by Pflug and Odlaug (69). The third step in the design of a BI system is the actual evaluation of the sterilization process in destroying the BI employing a full load of product. Process parameters (temperature, gas concentration, humidity, radiation dose) are established during this step. Finally, a determination is made either of the amount of (log cycle) reduction required for the desired degree of probability or of the level of microorganisms to be used as a BI to validate the sterilization process, qualify the sterilization vessel, and, subsequently, monitor the sterilization process (70).

Environmental Control

Sterility tests should be performed in a test area that conforms to Class 100 conditions as described by Federal Standard Number 209E (71). Class 100 conditions mean that no more than 100 particles per cubic foot of size 0.5 μm or greater, as measured by electronic particle counters, shall be found in the measured area. A comparison of the classes of air cleanliness is provided in Table 1.22. However, these classes refer to levels of particulate matter, not viable microorganisms. The 1987 Guideline on Sterile Drug Products Produced by Aseptic Processing recommends no more than 25 organisms per 10 cubic feet of air for a Class 100,000 area, and no more than 1 organism per 10 cubic feet for a Class 100 area. No recommendation for a Class 10,000 microbial specification is made in the docu-

ment, though a value of organisms per 10 cubic feet is common.

Great strides have been made in recent years to help ensure that Class 100 conditions are met and that adequate microbial monitoring is effected in a sterility testing facility. Probably the greatest advancement was the discovery by Whitfield (72) in 1961 of the concept of laminar air flow.

Laminar Air Flow

Phillips and Miller (73) have succinctly described the concept of laminar air flow (LAF). The employment of LAF cabinets, workbenches, and rooms in the proper execution of the sterility test and other aseptic operations is essential. The air emitted from LAF equipment is claimed to be 99.97% free from microbial contamination. This level is based on the removal of dioctylphthalate particles 0.3 μm and larger. Thus, although a theoretical 0.03% contamination level exists when using LAF equipment, the air within the confined area of the workbench or cabinet is considered to be sterile.

LAF equipment can deliver clean air in a vertical, horizontal, or curvilinear direction. The principles of vertical and horizontal air flow are shown in Figs. 1.10 and 1.11, respectively. Room air is taken into the equipment and passes through a prefilter, which removes large-size air contaminants. A blower then forces the prefiltered air through a second filter system in the LAF unit called a High Efficiency Particulate Air (HEPA) filter (Fig. 1.11). Air passing through the HEPA filter not only is 99.97% particle free, but also moves with uniform velocity along parallel flow lines. Proper aseptic procedures are to be practiced while working at the laminar flow workbench during sterility.

Quality control procedures must be adopted to evaluate and monitor the quality of the LAF hood environment. This includes monitoring with particles and microorganisms. Since LAF hoods are supposed to provide Class 100 air, they should be certified that this standard is met. Certification is done immediately after installation of new HEPA filters and at peri-

Table 1.22 Guidelines for Air Cleanliness Classes

Type of facility	Class 100	Class 10,000	Class 100,000
Laminar air flow			
Vertical flow room, vertical flow curtain units, vertical flow bench	Entire work area meets requirements at normal working height locations	Entire area meets requirements	Entire area meets requirements
Crossflow room, tunnel room, wall-to-floor room, crossflow bench	First work locations meet requirements	Entire work area meets requirements if particle generation, work locations, and personnel are reasonably controlled.	Entire area meets requirements
Nonlaminar air flow			
Conventional clean room	Will *not* meet requirements under operation conditions	Can be upgraded to meet requirements by placing laminar air flow devices within the room and continously filtering the recirculating air. Personnel and operation restrictions and janitorial maintenance are also required.	Will meet requirements with strict observation of rules governing personnel, operations, garmenting, and janitorial procedures

Rooms containing computer systems	Will meet requirements with personnel restriction and janitorial maintenance	Entire area meets requirements.	Entire area meets requirements
Maximum number of particles per cubic foot that are 0.5 μm and larger		Class	Maximum number of particles per cubic foot that are 5.0 μm and larger
100		100	0
1000		1000	7
10,000		10,000	65
20,000		20,000	130
100,000		100,000	700
1,000,000		1,000,000	6,500

Source: Liberty Industries, East Berlin, Connecticut.

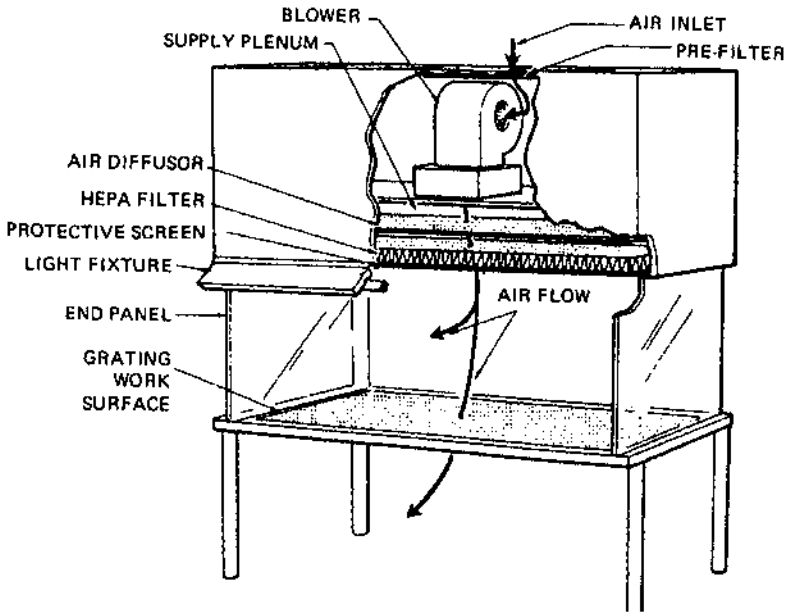

Fig. 1.10 Vertical laminar air flow bench (courtesy of Liberty Industries, East Berlin, Connecticut).

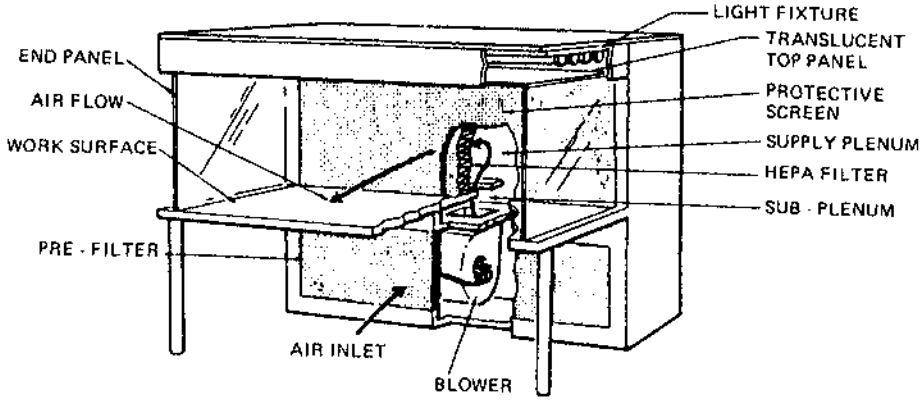

Fig. 1.11 Horizontal laminar air flow bench (courtesy of Liberty Industries, East Berlin, Connecticut).

odic intervals, usually every 6 months. The velocity of HEPA-filtered air is measured using an air velometer. Air velocity in all parts of the filter should be 90 ± 20 feet per minute. Air quality is evaluated using particle counters, microbial air samplers, and agar settling plates. The efficiency of the HEPA filter in removing particulate and microbial contamination is evaluated by employing the dioctylphthalate (DOP) test. This is a universally acceptable challenge test for HEPA filters. DOP is a volatile liquid that, under pressure, converts to a vapor or smoke having a size range of 0.28 to 0.4 μm. The DOP smoke is introduced at the supply plenum. A photometer probe then scans the entire HEPA filter surface. Any leaks in the filter will permit the DOP smoke to escape, and this will be detected by the photometer. Several references are available that describe the testing of laminar flow equipment (75–77). Phillips and Runkle (78) have published a comprehensive review of the biomedical applications of LAF.

For most sterility testing operations, horizontal LAF units appear to be superior to vertical flow hoods because the air movement is less likely to wash organisms from the operator's hands or equipment into the sterility test media (73). However, the operator must be specifically trained on how to utilize the air flow properly. HEPA-filtered air will not sterilize the surface of a contaminated object. It will only maintain sterility or cleanliness of an already sterile or clean object. All surfaces of the LAF hood except the HEPA filter itself must be thoroughly disinfected before placing any item inside the hood. All materials must be disinfected prior to introducing such materials onto the surface of the LAF workbench. For example, all glassware, containers, and other articles with surfaces that are nonsterile must be wiped thoroughly with a disinfectant solution before placing these items in the LAF unit. Sterile materials enclosed in protective packaging, such as plastic bags contained in polyethylene outer pouches, disposable syringes, sterile scissors and forceps wrapped in aluminum foil, wrapped membrane filter units, and the like may be introduced into the LAF unit by removing the outer protective

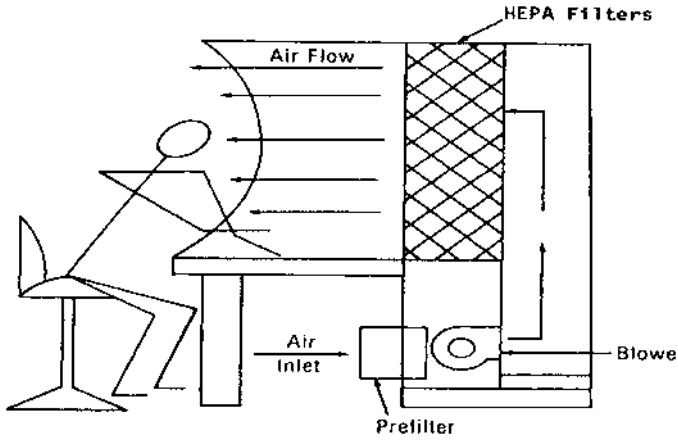

Fig. 1.12 Direction of air flow in a laminar air flow work station (courtesy of Liberty Industries, East Berlin, Connecticut).

package at the edge of the workbench before placing the sterile item on the workbench surface. Understanding of the LAF pattern is very important to avoid turbulence and blockage of HEPA filtered air reaching the critical work site (Fig. 1.12).

Design and Maintenance of Aseptic Areas

The USP states that the principal sources of contamination are the air and water in the aseptic processing area and the personnel, materials, and equipment involved in the processing. Avis (79) has described considerations that must be met in the design, construction, and implementation of a sterile products facility. An encyclopedia now exists for the design, operation, and all other aspects of clean rooms, white rooms, and sterile rooms (80). All areas must be designed and constructed to permit adequate cleaning, efficient operation, and comfort of personnel. Process flow must follow a plan by which product and personnel move to increasingly clean environments. An example of a process flow diagram is found in Fig. 1.13 (79).

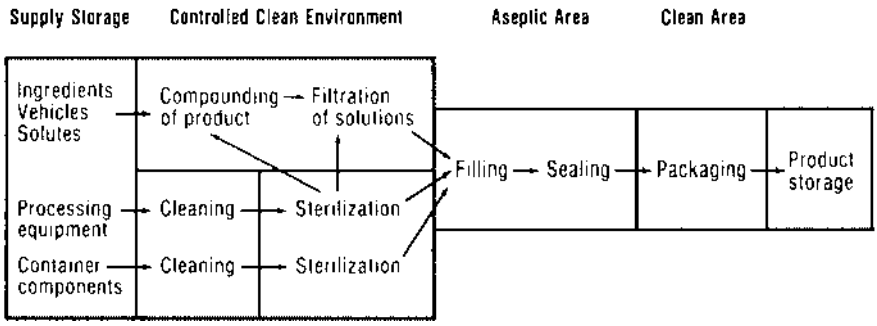

Fig. 1.13 Diagram of flow of materials through a production area (from Ref. 79.)

Ceilings, walls, and floors in the aseptic processing area must be sealed for ease and thoroughness in washing as well as treatment with disinfecting products. All counters, cabinets, and sinks should be constructed of stainless steel. All equipment, service lines and facilities, and other essential room fixtures should be constructed in such a manner to permit ease of cleaning and disinfecting and to prevent the accumulation of dust and dirt.

Several engineering features of a well-designed aseptic area are listed in the USP. However, newer proposals emphasize the principles involved rather than describe in detail the features of the facility. The following are a number of features, not all of which are necessarily applicable to any particular facility:

1. Hoods, cabinets, and other enclosures to serve as a primary barrier around the process, while the aseptic room itself serves as a secondary barrier
2. Maintenance of differential positive air pressures to prevent inward leakage of air
3. Effective filtration of air supplied to the primary and secondary barriers
4. Provision of air locks and/or air showers at the entrances

to rooms, a gowning room, and adequate space garment storage for personnel
5. An effective intercommunication system and a suitable room arrangement to minimize traffic

An appropriate program should be established initially to qualify aseptic areas and equipment and routinely to monitor the integrity of the measures. The least controlled and potentially greatest source of contamination originates from the people working in the aseptic processing area and conducting the sterility test procedures. Training of personnel to minimize potential contamination arising from people is discussed in the section, "Personnel Training."

Methods of Evaluating the Environment

A number of proven, quantitative viable and nonviable microbial counting methods are available for evaluating and monitoring the environment in which sterility tests are conducted. These methods can be further divided into air- and surface-sampling methods.

Air-Sampling Methods

Slit-Air Sampler. This is a device that collects viable airborne microbial and particulate contamination (see Fig. 1.14). A 150-mm sterile agar plate containing a layer of sterile agar, usually trypticase soy agar, is placed on a circular plate in the slit-air device, and the cover containing the slit is secured above the agar plate. The speed of the rotation of the plate and the volume of air sampled can be adjusted to record the desired rate and degree of contamination of the air environment. The split-air sampler is one of the most widely used monitoring methods for sterile manufacturing and quality control environments.

Liquid Impinger. This device works by using a vacuum source to suck in air at a high velocity through an isotonic impingement fluid, then passing the fluid through a membrane filter by vacuum and incubating the filter on an agar plate. While liquid impingement thoroughly collects viable mi-

Fig. 1.14 Slit-to-agar biological air sampler. (Courtesy of New Brunswick Scientific Co., Inc., Edison, New Jersey.)

crobial contamination within a given cubic foot of air, it suffers two primary disadvantages. One disadvantage is the fact that microbial counts may be underestimated because the high velocity of impingement kills many organisms on impact with the agar surface. The other disadvantage is the problem of air locks occurring at the filter surface as the impingement and diluting fluids are being vacuum filtered.

Electronic Air Particle Counters. These instruments count all particles in the environment and cannot differentiate between viable and nonviable particles. These counters are especially useful in determining the number of particle counts per cubic foot to classify the cleanliness of a particular room or area.

Settling Plates. These represent the simplest means of evaluating the microbiological quality of air. A 100-mm petri dish containing trypticase soy agar or other suitable medium

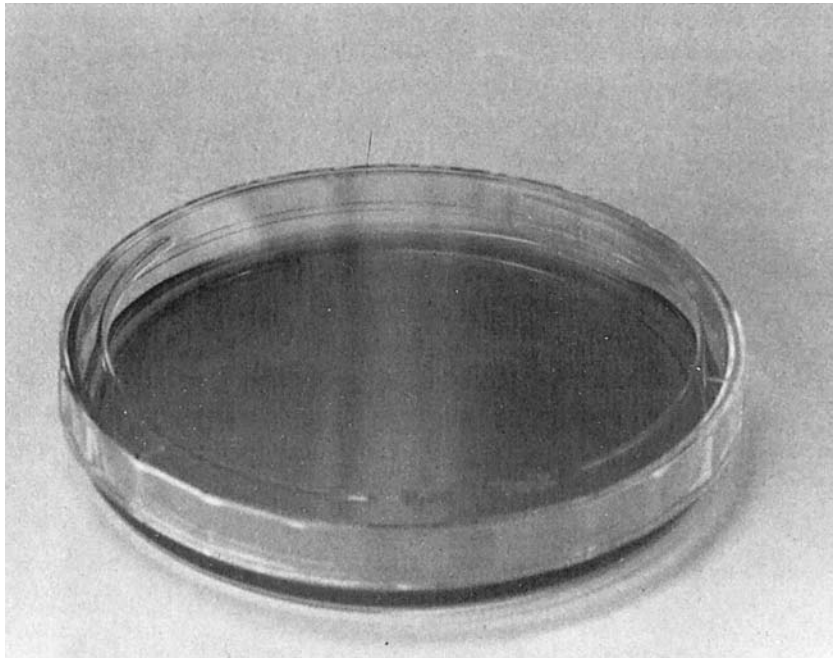

Fig. 1.15 Agar settling plate.

is placed in a sampling location with the lid removed as shown in Fig. 1.15. The period of sampling is controlled—usually 2 to 4 hours—before the lid is placed over the medium and the plate incubated. Colonies are counted, and many different locations within the sterility testing and manufacturing areas can be controlled and compared for microbial contamination. The major disadvantage of this method is that the volume of air sampled and represented on the agar plate is unknown.

Centrifugal Air Sampler. This device (see Fig. 1.16) is another method used to determine airborne contamination. Airborne microbes approximately 16 inches above the sterile drum housing are drawn toward the impeller blades. Then, owing to the applied centrifugal force, 4014–4178 rpm, the microbial particles are impacted at high velocity onto the agar surface of the agar strip wound around the impeller blades.

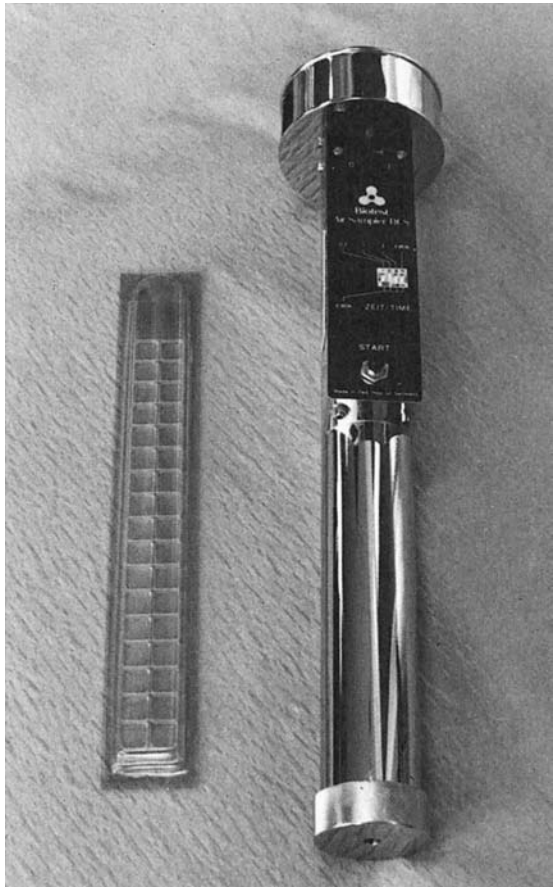

Fig. 1.16 Biotest RCS centrifugal air sampler.

After incubation, colonies on the strips are counted; the results reported as colony-forming units per unit volume of air. The air capacity of the sampler per minute is 40 liters. In a comparative study of airborne microbial recovery rates (81), the RCS centrifugal air sampler was found to be significantly more efficient than the slit sampler and the liquid impinger. The centrifugal air sampler samples a greater area (1.2 cubic feet) versus 0.5 cubic feet sampled by the slit sampler (82).

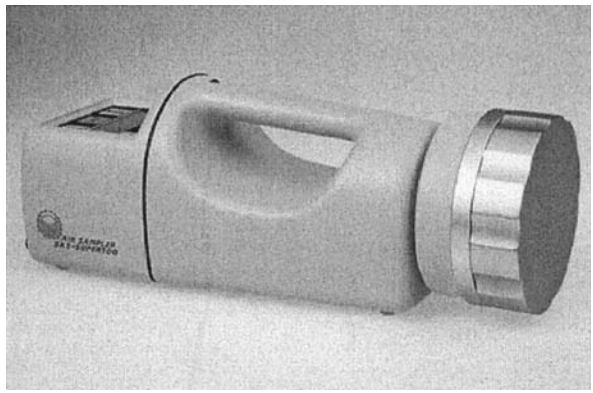

Fig. 1.17 SAS® air sampler (courtesy of Bioscience International, Rockville, Maryland.)

Sieve Impaction Air Sampler. A relatively new development in air sampling is a class of samplers known as the sieve impaction air sampler (see Fig. 1.17). Air is drawn through the sieve toward a cartridge holding nutrient medium. Any organism present in the air sample will be deposited on the agar surface of the cartridge. After the sample is collected, the cartridge is removed and incubated. After incubation, colonies are counted; the results are reported as colony-forming units per volume of air. These units can typically sample a relatively large volume (up to 1000 liters) in as little as 8 minutes.

Surface-Sampling Methods

Rodac Plates. These are specially built petri plates in which sterile culture media, usually trypticase soy agar containing Polysorbate 80 and lecithin, is poured onto a baseplate until a convex surface extends above the rim of the baseplate (see Fig. 1.18). Once the molten agar has solidified, the agar surface can be gently pressed against a selected surface, for example, the surface of an LAF workbench. The lid is replaced, and the plate is incubated for the required length of time at a controlled temperature. Surface contamination can be quantified by counting the colonies after incubation. The presence

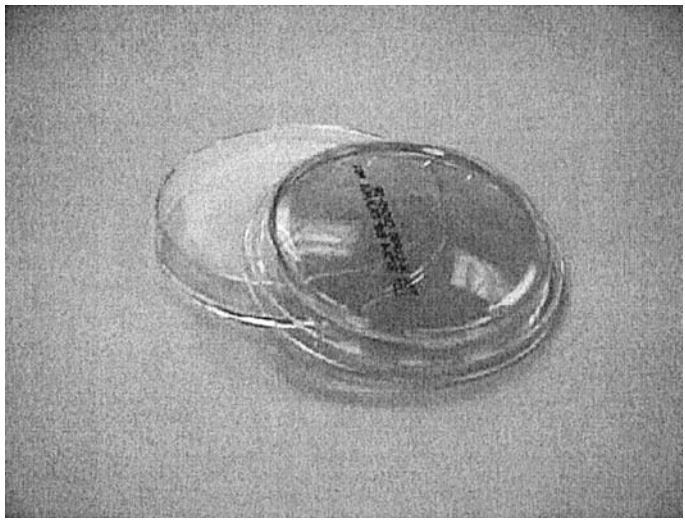

Fig. 1.18 Convex surface of Rodac agar plate.

of Polysorbate 80 and lecithin serves two purposes: to aid in the complete contact and removal of microbes from the sampled surface and to permit cleaning of the sampled area with water and/or a disinfectant solution.

Swab-Rinse Test. This is a simple surface sampling method employing sterile cotton swab tips to sample locations that are unwieldy for Rodac plates or difficult to reach. The swabs are then placed into tubes of culture media or, for microbial quantification, are mixed with sterile water and then a sample of the water is placed on a solid agar plate.

Many of these environmental testing procedures and a suggested program for determining microbiological burden and action levels for both nonsterile and sterile environments can be found in an article by Dell (83). Tables 1.23 and 1.24 are reproduced from Dell's article. These guidelines can be useful in establishing a program design for environmental monitoring specific to the history, conditions, and needs of any particular manufacturing and sterility testing facility.

Table 1.23 Example of an Environmental Testing Program for Monitoring a Sterile Production Facility

Element	Membrane filtration	Most probable number	Pour plate	Rodac	Swab	Settle plate	Slit-air	Other[a]	Frequency[b]
Walls and floors									
Sterility test area				X	X				M
Controlled areas				X	X				M
Critical areas				X	X				M
HEPA-filtered air									
Controlled areas						X	X	DOP	M
Critical areas						X	X	DOP	B
Sterility test area						X	X	DOP	B
Components	X	X	X					MLT	H, B
In-process bulk	X	X	X					MLT	H, B
Production equipment									
Tanks					X				
Hoses and lines	X							DM, VF	M
Filling equipment								VF	
Compressed air and gas	X							ORG	H
Potable water	X	X	X					LF	D
Deionized and distilled water	X	X	X					PT	D
HEPA filter						X	X	V, DOP	M
Finishing supplies	X		X						H

Source: Ref. 83.

[a] DOP = dioctylphthalate smoke test; DM = direct method sterility; LF = lactose fermentation (standard methods of analysis); MLT = USP microbial limits test; ORG = organic material (oil); PT = pyrogen test; V = velometer; VF = vial fill or media fill test.

[b] B = batch or shift; D = daily; H = history; M = monthly.

Table 1.24 Example of Guidelines (Action Levels) for Environment Monitoring of Sterile Production Elements

Sterile processing location[a]	Bacteria (per cm^2)		Mold (per cm^2)	
Controlled areas (Class 10,000–100,000)				
Walls, floors, equipment; swab or Rodac	<1		<1	
Assembly rooms	<10		<2	
Critical areas (Class 100)				
Walls, floors, equipment; swab or Rodac	0		0	
	SP	SA	SP	SA
Controlled areas—air sampling[b]				
Component preparation areas	10	100	2	25
Transfer areas	2	25	1	10
Gowning rooms	2	25	1	10
Wash booths	5	50	2	10
Staging areas	50	500	10	100
Compounding rooms	2	25	1	10
Critical areas—air sampling[b]				
Filling rooms	2	10	0	2
Sterility test laboratory	1	<1	0	0
Water[c]		<1/ml	0/ml	

Source: From Ref. 83.

[a] Controlled areas: sampled following sanitizing and prior to use.

[b] Air sampling: settling plate exposed for 30 minutes or slit-to-agar sampling at a rate of 28.3 liters air/minutes for 30 minutes.

[c] Water tests same as for nonsterile manufacturing. Microbial count refers to "at time of use."

The ultimate purpose of environmental control of microbial contamination is to minimize the potential for inadvertent product contamination. The less the potential for contamination, the greater the assurance that the product is sterile. The sterility test then can be used primarily as a confirmation of the sterility already built into the product.

A 1990 FDA 483 observation was issued to a manufacturer who released a lot of product based on the fact that it passed a sterility test although environmental monitoring during production of the sterile bulk drug revealed objectionable conditions. In another case, a manufacturer decided to release a lot of product based on a successful retest of the sterility test because the contaminant that caused the failure of the original sterility test was not isolated on any environmental sample in either the production area or the sterility test laboratory. However, the FDA countered that environmental monitoring provides only a "snapshot" of the environment, and that it is not surprising that different media and different "sampling" times will selectively identify different organisms.

Personnel Training

Most inadvertent contamination found in the sterility testing of parenteral products originates from the personnel involved in the testing program. Nearly all personnel-induced accidental contamination is produced either by the ignorance of an individual who has not been adequately trained in good aseptic technique or by the carelessness of an individual who has been trained in good aseptic technique. Thus, learning and applying aseptic technique not only requires physical and intellectual abilities, but also involves the development and persistence of a correct mental attitude. The latter is very difficult to instill. No one in a free society can be forced to comply to rigid standards. Supervisors who hire personnel to perform sterility tests should abide by three general rules:

1. The supervisor must recognize the need to comply with strict aseptic technique.
2. The supervisor should hire people who are willing to be trained and to accept and follow aseptic procedures.
3. The supervisor must effectively communicate and exemplify the importance of adhering to aseptic technique without breeding ill feelings and subsequent poor attitudes.

Current good manufacturing procedures (CGMPs) (Section 211.25) contain several statements regarding the training of people engaged in the manufacture, processing, packaging, and holding of drug products. Personnel will be trained not only to perform sterility tests, but also to understand CGMPs and standard operating procedures as they relate to sterility testing.

Training in correct aseptic technique includes five general areas of education:

1. General rules to follow when a person is working in a clean or sterile room
2. Proper gowning technique
3. Proper use of the LAF workbench or other clean environment
4. Specific operations and manipulations while actually performing the sterility test that are essential in maintaining asepsis
5. Proper cleanup at the conclusion of the test

No matter how well constructed a sterile or clean room may be, it cannot compensate for people working in the area who are untrained with respect to sources of contamination. DeVecchi (84) has published 29 rules or restrictions concerning when training people to work in sterile environments. These are listed in Table 1.25.

The importance of gowning may be emphasized best by reference to a statement by Abdou (85):

> A room in which people work cannot be made sterile, regardless of how closely instructions concerning personal hygiene are followed. Twenty percent of the cutaneous flora is located so deep within the follicular channels that it cannot be reached by normal disinfection procedures. Such a reservoir of organisms ensures that the surface flora will quickly reestablish itself after the usual treatment of the skin with disinfectants. The epidermal frag-

Table 1.25 General Rules and Procedures for Working in a Sterile Environment

1. Before entering any sterile environment, personnel should understand the responsibilitites of their position and know clean-room techniques and system operations.
2. Personnel must react effectively in emergencies such as: fire outside or inside the sterile room, explosions outside or inside the sterile room, electrical failure, breaking of containers holding toxic or nontoxic substances, illness or injury.
3. Everyone who enters the sterile area must be familiar with gowning technique.
4. Without exception, all personnel working, supervising, controlling, or maintaining a sterile room should wear the approved sterile-room garments.
5. No sterile-room garment may be used a second time without being rewashed and resterilized.
6. Everyone working in sterile areas must know the disinfection and sterilization procedures.
7. Once inside a sterile room, personnel should avoid returning to the air lock. If a worker must go to the restroom, complete resterilization and regarmenting are necessary prior to reentering the clean room.
8. Plastic bags for disposal of used garments should be provided in the air locks adjacent to powdered-antibiotic filling or preparation areas. The garments may be transported in these bags to the laundry area without risk of cross-contamination between product and personnel.
9. For reasons of comfort and efficiency, establish a minimum number of people to be allowed in the air locks at any one time.
10. No personal articles (purses, bags, etc.) are permitted inside the sterile rooms or air locks.
11. No one who is physically ill, especially with a stomach or respiratory disorder, may enter sterile rooms or sterile areas.
12. All verbal communication with people outside of the sterile room should be accomplished through use of the intercom—never through air locks or passthroughs.
13. The sterile-room doors must be kept closed at all times. They may open only to admit one person or product at a time.

Table 1.25 Continued

14. Smoking is prohibited inside sterile rooms and neighboring rooms.
15. The use of cosmetics, wigs, makeup, long nails, rings, watches, etc., is prohibited in sterile rooms.
16. All materials, containers, or equipment introduced into the sterile room must be subjected to stringent sterilization procedures prior to entering the sterile areas.
17. Only long-fibered materials may be used for cleaning in sterile areas. Synthetic materials are suggested. Mops, brooms, and other customary cleaning equipment should not be used in sterile areas.
18. Paper in any form (except paper produced expressly for sterile-room standards, and meeting Class 100 conditions as delineated in Federal Standard 209B) is not allowed in sterile rooms.
19. Under no circumstances should food or beverages be introduced into a sterile room.
20. No pencils or ball-point pens should be used in a sterile room. Magic markers or felt-tip pens are suggested.
21. When it is necessary that paper forms be used in sterile areas, the form should be shielded with a clean plastic covering that has a window exposing the area on which the operator is writing.
22. Two different products are not to be processed in the same sterile room at the same time.
23. Antibiotic products in a powder form or liquid products of any kind should be manufactured in areas designated specifically for that purpose.
24. Disinfection and cleaning of the room must be completed at scheduled times. All personnel in the sterile room should know the cleaning and disinfection techniques used.
25. The sterile room must be kept clean at all times. Personnel, equipment, and materials introduced into a sterile room should be kept to a minimum.
26. Once production runs are discontinued, any material from the previous production run should be removed from the sterile room to avoid cross-contamination.

Table 1.25 Continued

27. Cleaning and/or disposal of all support material should be done after each workday.
28. Sterile-room furniture should be of simple design. No chair covers to chairs with foam parts are allowed in sterile areas. Tables should be stainless steel and without drawers. Equipment should be properly covered. No equipment with belt-driven or high-speed moving parts should be permitted in a sterile environment unless that equipment has proper covering.
29. All materials, containers, equipment, etc., authorized for sterile-room use must be labeled so as to be easily identified by clean-room personnel.

Source: From Ref. 84.

ments that people shed carry microorganisms, and the more vigorous the physical activity, the more the shedding. The skin of a healthy adult can shed between two and six million colony-forming units in one-half hour of vigorous activity.

Thus, the use of low or particulate gowning materials and adherence to strict gowning procedures will help to ensure that the human body and clothing will not be a source of contamination. However, Brier et al. (86) reported that employment of clean room gowning did not affect the contamination rate of admixtures compounded in a hospital pharmacy. What was important was that intravenous admixtures were compounded using a LAF workbench.

Proper use of the LAF working environment in the content of this discussion refers to the movement and manipulations of hands and objects in the hood without interfering or interrupting the flow of sterile air onto articles that must be kept sterile. Procedure 8 in Appendix IV should be reiterated at this point. Opening containers, devices, or other articles in which a sterile surface or pathway will be exposed should be completed so that the sterile part faces the HEPA-filtered air.

Moving, tilting, or otherwise manipulating open containers must be accomplished without fingers and hands either making contact with the exposed opening or coming between the opening and the airstream pathway. Sterility test aids such as sterile forceps, scissors, filters, and other devices must be handled with care so as not to touch-contaminate the article. Whenever the operator suspects that a sterile surface has accidentally been touched, that article should be discarded. Fingers that have been disinfected and subsequently make contact with a nonsterile object should be disinfected again with a suitable disinfectant solution or foam. The most important aspect of working in an LAF workbench is mental concentration on the task at hand, always realizing where the hands are in relation to the HEPA-filtered airstream and the critical work sites.

Operator training on the actual sterility testing procedures means the learning of the standard operating procedure (SOP) written for the sterility test to be executed. This step is probably the most time-consuming component of the training process. The operator will work closely with an experienced supervisor or other trainer for the length of time required for the operator to learn the SOP and perform the test without error in procedure and/or technique. The rate of false-positive sterility test samples will be ascertained for each new operator; obviously, a certain acceptable rate must be attained for the operator to be entrusted with future sterility test responsibility. Each sterility testing facility should set up a monitoring program to check periodically the rate of false-positive samples produced by each operator. Hospital pharmacies that prepare intravenous admixtures and other sterile products should also maintain training programs for their sterile products technicians. Organized training programs based on national standards have been considered by the American Society of Hospital Pharmacists (87).

Regardless of how well a person is trained in the procedural aspects of conducting sterility tests, that individual must also possess the right mental attitude toward the respon-

sibility and implications at hand. Otherwise, a mediocre or poor attitude will result in carelessness, indifference, and, ultimately, errors in technique. A right attitude must be present in the individual at the beginning and then maintained and motivated through supervisory encouragement and reward.

ALTERNATIVES TO THE COMPENDIAL STERILITY TEST

The limitations of the USP/NF sterility test have already been addressed. They include the large sampling error due to the very small sample size tested for sterility, the problem of inadvertent contamination during sterility testing, and the difficulty in recovering low-level contamination; these all contribute to reasons for finding alternative procedures to the USP test as it is described for a reference test. Another reason for searching for alternative sterility test procedures is to fill the need of hospital pharmacies and other laboratory environments in which sterile solutions are prepared or manipulated in some manner. The USP referee sterility test is too time consuming and costly to be used routinely in hospital practice, especially with the enormous numbers of intravenous admixture solutions being prepared.

At least two basic methods have been used for sterility testing in hospital practice. One involves the sampling of an aliquot volume of solution from an intravenous bottle (45), while the other method involves the filtration of all of the remaining portion of the contents of the bottle through a closed filter system (88). In the first method, an aliquot sample is added to a concentrated broth solution such as double-strength brain-heart medium, FTM, or other suitable culture medium, or if it is feasible, the concentrate is added in a volume equal to the contents of the bottle as it is. The container is incubated and then inspected for the presence of microbial growth. The advantage of this method is its simplicity and cost. Its disadvantages include its potential for accidental contamination and the inability of one culture medium to promote the growth of all potential microbial contaminants, especially

in large-volume solutions because of the high dilution factor. In the second method, a special device, such as Steritest (Millipore; Figure 1.19) or IVEX (Abbott; Fig. 1.20), is designed to permit the filtration in a closed system of the entire contents of a bottle through a plastic presterilized unit containing a 0.22–0.45-μm membrane filter. TSB or FTM is then added aseptically, and the unit is incubated intact. This closed system was designed to reduce the rate of false positives and to

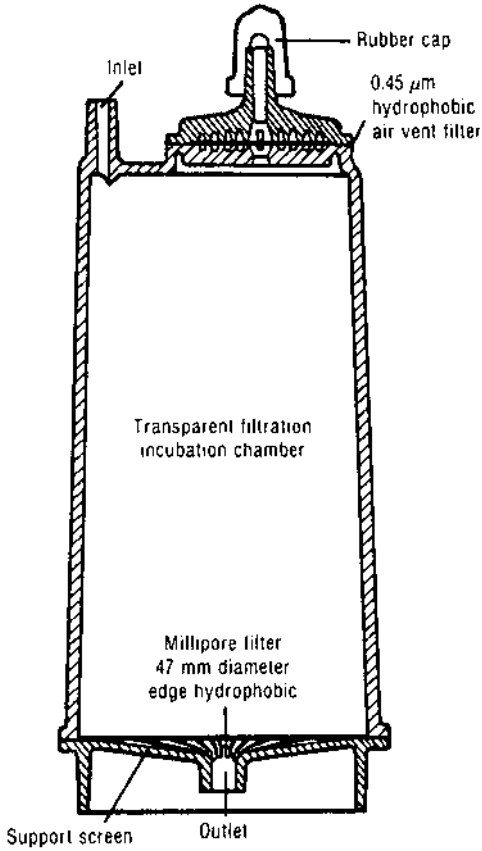

Fig. 1.19 Steritest chamber (Millipore Corp., Bedford, Massachusetts, from Ref. 78).

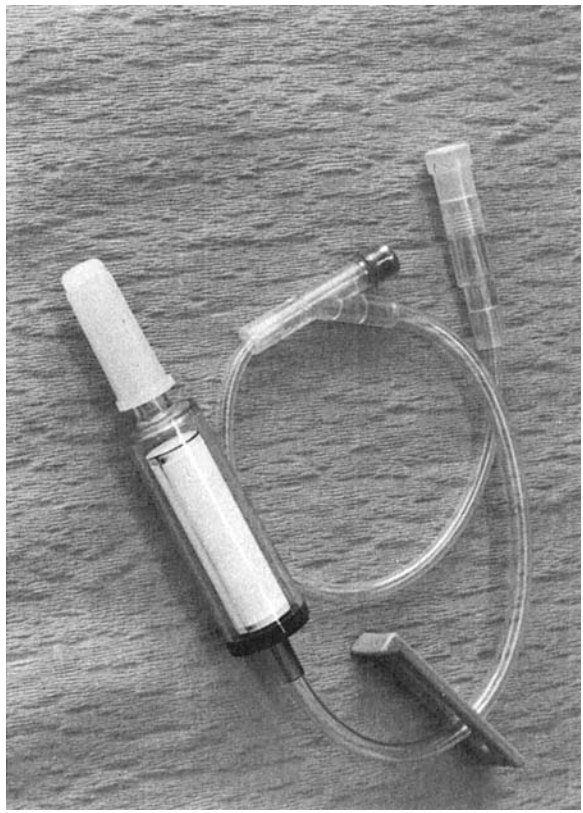

Fig. 1.20 IVEX-2 device (courtesy of Abbott Laboratories, North Chicago, Illinois).

provide a more convenient method of sterility testing large-volume solutions and admixtures in hospitals (89). Disadvantages of the filter device system are its relatively high cost and some concern about its sensitivity to low-level contamination.

Posey et al. (90) compared these two methods for detecting microbial contamination in 1-liter plastic bags of parenteral nutrition solutions containing 1 and 1000 bacterial or yeast organisms per milliliter. From each bag, 10-ml aliquots were withdrawn and injected into blood culture bottles. The

remaining fluid was filtered through the Addi-chek system. The aliquot sampling method consistently detected each of the organisms tested at levels of 100 organisms per liter and above. The filtration method consistently detected all levels of contamination. The authors concluded that the aliquot sampling method was inexpensive and easy to use, but failed to detect some contaminated solutions. The filtration method detected all levels of contamination, but is more costly in both time and money, and its reliability needs additional assessment.

The Addi-chek system was compared in a like manner with the IVEX-2 Filterset in the sterility testing of intravenous admixtures (91). Both filter systems were comparable in detecting low-level microbial contamination, but the IVEX-2 system can be used to test for contamination when used as an in-line filter for patient administration of intravenous fluids. Like the aliquot sampling method, the IVEX-2 system is less expensive than the Addi-chek system. Both IVEX-2 and the method of combining equal volumes of product sample and double-strength TSB were found to be more reliable and sensitive than Addi-chek in detecting low-level bacterial contamination in intravenous solutions, especially Dextrose 5% (92,93). Addi-chek sterility testing consumed more time in processing, allowing dextrose 5% time to exert an inhibitory effect on microbial growth. Also, Addi-chek uses a 0.45-μm membrane filter, while IVEX-2 contains a 0.22-μm filter.

A modification of the Addi-chek device to be used as an alternative to the MF method for the sterility testing of antibiotics has been described (94). The modified unit is shown in Fig. 1.18. Two separate spikes are connected to a two-way valve that prevents the siphoning of the antibiotic into the rinse-and-media line. The rinse-and-media line is used to transfer the rinse solution to the canister. The rinse removes inhibitory residual antibiotic from the canister so that the contaminating microbes that may have been deposited on the filter may grow. After the rinse procedure, one canister is filled with TSB and the other with FTM. This system has been found

to recover organisms with equal efficiency (as compared with the USP membrane filtration method) and, since it uses a closed, presterilized, and ready-to-use system, greatly reduces the chances of operator error and accidental contamination.

The use of an in-line 0.22-μm membrane filter set to test the sterility of intravenous solutions and administration sets (under actual use conditions) was found to have valuable application in intravenous fluid administration (95). Following filtration, brain-heart infusion broth was introduced into the filter chamber, and the filter sets were incubated. Microbial contamination was found for all contaminated intravenous solutions and administration sets. No false-positive results were found.

Sterility testing of small-volume unit dose parenteral products in the hospital environment was suggested by Rupp et al. (96), who used a sterile filter unit like those already described. For each lot of unit dose syringes, 10% were filtered before adding FTM to the filter unit. The units were incubated and inspected for turbidity or color change.

A practical hospital sterility test method for monitoring intravenous admixtures combines the MF technique and fluorescent microscopy (97). Solutions are filtered under vacuum through a 0.6-μm membrane filter. Then, a staining compound (acridine orange solution) is poured onto the membrane and allowed to stand for 3 minutes. The stain is removed by vacuum, and the membrane is removed and mounted on a microscope slide. The membrane is examined within an hour with a light microscope fitted with an epifluorescent illuminator system. Any bacteria entrapped on the membrane surface will react with the fluorescent stain, and when illuminated with incident light, the total number of cells can be counted. The correlation between fluorescent counts and plate colony counts is excellent, although the counts determined by fluorescence microscopy took only an hour or less, while plate counting requires a 48-hour incubation. Sensitivity of this new method can be increased to levels as low as 25 organisms per milliliter, and the technique can be automated.

Several alternative methods of sterility testing have been suggested for which neither aliquot sampling nor filtration is employed. One method suggested the addition of dehydrated broth powder (thioglycollate medium) to a random selection of bottles from each batch of infusion fluids before sterilization by autoclaving, followed by incubation and daily inspection for turbidity (98). Another method employed the use of an electronic particle counter to detect contaminated culture media within 24 hours after adding contaminated membrane filters to the media (99). There is also the luciferase assay for adenosine triphosphate (ATP) since detection of ATP indicates the presence of living cells (100). Each of these proposed sterility test alternatives offers one or more distinct advantages over the USP/NF sterility test either in terms of convenience, reducing the incidence of inadvertent contamination, or in significantly reducing the time required for detection of contaminated products. However, they are not necessarily alternatives to be preferred to a referee test in an advisory situation. Only in certain situations might one of these methods be a preferable alternative to the USP sterility test, especially in hospitals. For example, the luciferase ATP assay presents a very rapid method that can be used in septicemia investigations in which a large number of intravenous fluids must be tested as quickly as possible.

Several manufacturers expressed the desire to employ an automated sterility testing system. One commercially available system is the Bactec system (Johnston Laboratories). Its principle of operation is based on the detection of radioactive gas produced by decomposition of labeled substrates by microbial action. Samples of pharmaceutical product are withdrawn, inoculated into Bactec culture vials containing ^{14}C substrates, and incubated for 2–5 days. The Bactec instrument automatically tests the vials by analyzing the atmosphere in the vials. If the vial contains microorganisms, they will metabolize the ^{14}C substrates to product $^{14}CO_2$. A positive result will be indicated once a threshold level of $^{14}CO_2$ is exceeded. Models are available to test 60 culture vials per hour. The system of-

fers expediency and convenience not characteristic of official sterility testing methods.

An article by Lewandoski cites several reasons for the lack of an approved rapid sterility test (107).

1. Most rapid methods do not allow for subsequent culture and identification in the event of a positive sterility testing result. The identification of a positive sterility test result is key to the investigation (mentioned above).
2. No rapid method is currently sanctioned by any major pharmacopeia. The USP, EP, and Japanese Pharmacopeia (JP) all cite the traditional sterility test as the final release test for injectable products.
3. Legal issues—lack of a compendial sterility test is difficult to defend in court.
4. False positives—some rapid methods may detect dead cells.
5. The traditional sterility test has been employed in the pharmaceutical industry for more than 40 years.
6. To date, no pharmaceutical company has submitted an NDA with a rapid method as a release test (e.g. no one is willing to "go first"). A representative from CDER is cited in the article: "For a long time, I've been hoping to see rapid methods in applications [NDA] where the test is par of the product release specification." The coming years will be very interesting as rapid methods develop.

Another alternative to the sterility test is the concept of *parametric release*; the sterility test is waived in lieu of a validated terminal sterilization cycle capable of a high sterility assurance level. Parametric release can only be employed when there is assurance that any article processed will meet the requirement for the sterility test.

Four methods of sterilization that qualify for parametric release: moist heat (steam), dry heat, ethylene oxide, and ionizing radiation. These methods of sterilization are recognized by U.S. and European regulatory bodies (see USP <1222>

and EMEA Note for Guidance on Parametric Release, respectively).

There are several requirements in order for a product to qualify for parametric release:

1. The manufacturer must obtain authorization from the appropriate regulatory body or bodies prior to initiation.
2. The terminal sterilization method must be designed and validated to achieve a sterility assurance level of at least 10^{-6}.
3. The microbial load of the article prior to sterilization should be quantified and characterized because the microbial load and type(s) of organism(s) present must be considered for the terminal sterilization cycle.
4. The use of physicochemical indicators is required in *every* terminal sterilization load. Indicators should be correlated to at least a 10^6 log reduction of an appropriate BI (see Table 1.21 for a list of appropriate BIs for various sterilization methods).
5. Verification that the established parameters of the terminal sterilization cycle were performed correctly.
6. Verification that the BIs were reduced to sterility after sterilization and incubation.

If any of the established parameters are not met or if the biological indicators are positive after incubation, then the lot must be rejected. If parametric release is employed, then the use of the sterility test to justify release of the lot is strictly forbidden.

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2

Pyrogen Testing

INTRODUCTION

When injected into humans in sufficient amounts, pyrogens will cause a variety of adverse physiological responses (Table 2.1). The most common or recognizable response is an increase in body temperature, from which the name "pyrogen" is derived (Greek *pyro* = fire, *gen* = beginning). Pyrogenic responses rarely are fatal unless the patient is very sick and the dose is very large. Nevertheless, pyrogens are considered toxic substances and should never be injected knowingly. Pyrogen contamination of large-volume parenteral (LVP) solutions is especially serious because of the large amounts of fluid administered to people whose illnesses must be severe enough to warrant the use of such large volumes.

Pyrogens come from microorganisms. All microbial forms produce pyrogen; however, the most potent pyrogen originates from gram-negative bacteria. The entity primarily involved in pyrogenic reactions in mammals is the lipopolysaccharide (LPS) from the outer cell membranes of gram-negative bacte-

Table 2.1 Adverse Physiological Effects of Pyrogens in Humans

Primary
Increase in body temperature
Chilly sensation
Cutaneous vasoconstriction
Pupillary dilation
Piloerection
Decrease in respiration
Rise in arterial blood pressure
Nausea and malaise
Severe diarrhea
Pain in the back and legs
Headache
Secondary
Cutaneous vasodilation
Hyperglycemia
Sweating
Fall in arterial blood pressure
Involuntary urination and defecation
Decreased gastric secretion and motility
Penile erection
Leucocytopenia, leucocytosis
Hemorrhage and necrosis in tumors
Altered resistance to bacterial infections
Depletion of liver glycogen
Rise in blood ascorbic acid
Rise in blood nonprotein nitrogen and uric acid
Decrease in plasma amino acids

ria (1). Another name for LPS is endotoxin. Although not entirely correct, the names pyrogen, LPS, and endotoxin are routinely used interchangeably. Figure 2.1 is a schematic representation of the three cell wall layers of a gram-negative microorganism (1). The outer membrane shown in the figure is not found in gram-positive bacteria. This structure contains the LPS moiety that interacts with the coagulable protein of

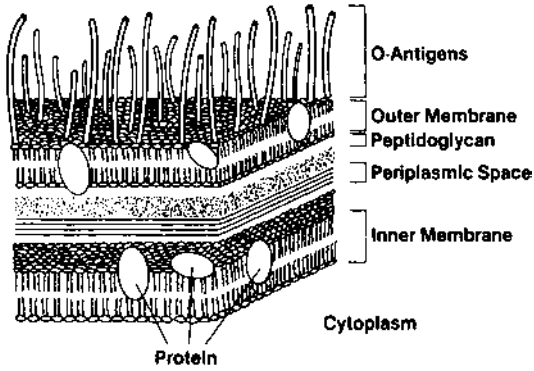

Fig. 2.1 Schematic representation of the three cell wall layers of a gram-negative bacterium. (From Ref. 1).

the amebocytes of the horseshoe crab, a phenomenon from which evolved the *Limulus* Amebocyte Lysate (LAL) test.

LPS, extracted and recovered as a colloidal suspension, may be split by mild acid hydrolysis into lipid A and degraded polysaccharides (2). Lipid A is composed of B-1, 6-glucosamine disaccharide units with â-hydroxymyristic acid replacing one of the amino hydrogens and fatty acids replacing hydrogen in some of the —OH groups (see Fig. 2.2). Each two glucosamine units are separated by two phosphate moieties, forming a linear polymer (1). Lipid A alone lacks biologic activity, yet LPS is toxic, probably because polysaccharide increases the aqueous solubility of lipid A. Kennedi et al. (3) showed that when lipid A is separated from the polysaccharide component of endotoxin, it loses more than 99.9% of its pyrogenic activity in rabbits.

Freedom from pyrogenic contamination characterizes parenteral products in the same manner as sterility and freedom from particulate matter. Preventing the presence of pyrogens is much preferred over removing pyrogens in parenteral products. Preventing pyrogenic contamination primarily involves the use of ingredients, solvents, packaging materials,

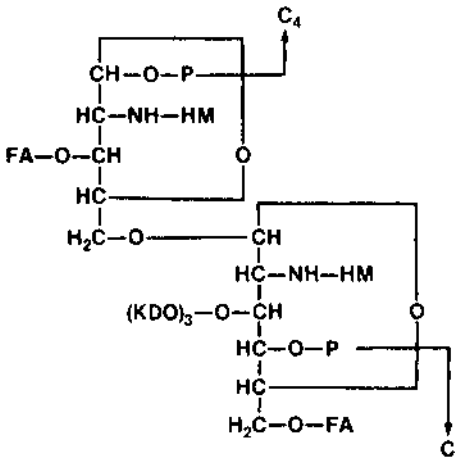

Fig. 2.2 Structure of unit of lipid A from *Salmonella* lipopolysaccharide. KDO: 3-deoxy-D-mannooctulosonic acid; HM: â-hydroxymyristic acid; FA: other long-chain fatty acids. (Adapted from Ref. 2 and Reitschel et al., *Eur. J. Biochem*., *28*, 166, 1972).

and processing equipment that have been depyrogenated initially, then employing correct and proper procedures during the entire manufacturing process to minimize the possibility of pyrogen development.

HISTORY

The pyrogenic response has been known since 1865, when it was reported that an injection of distilled water produced hyperthermia in dogs (4). Later, in 1876, the presence of a fever-producing substance, called a pyrogen for the first time, was found in extracts of putrefying meat (5). Identification of the pyrogenic component from bacteria was attempted by Roussy in 1889 (6) and Centanni in 1894 (7), who determined that the pyrogen was nonproteinaceous. Hort and Penfold (8) in 1911 made significant contributions in relating the production of fever and the administration of intravenous infusions. They also were the first to use rabbits as an animal model to study

the pyrogenic response. They showed that the incidence of chills and fever following intravenous injection could be reduced markedly if freshly prepared distilled water was used as the injection solvent. Investigators (9,10) related fever production in rabbits with the injection of bacterial culture extracts and showed that sterile solutions free from endotoxins did not cause the febrile response. Pyrogenicity seemed to be related to the gram stain reaction; gram-negative organisms produced a pyrogenic response, while gram-positive organisms did not. In addition, bacterial pyrogens were not destroyed by autoclaving or removed by filtration.

It is interesting to note that while the medical significance of a pyrogen was recognized during these years, it was not until 1923 that Seibert (11,12) recommended that all pharmaceuticals be tested for pyrogens. Seibert's carefully controlled experiments confirmed Hort and Penfold's (8) results using the rabbit as the animal model for detecting the presence of pyrogens in injectables. Seibert also demonstrated conclusively that pyrogens originate from water-borne organisms, and are heat resistant, filterable, and can be eliminated from water by distillation. Rademacher (13) substantiated Siebert's results and presented instructions for the preparation of pyrogen-free parenteral solutions. CoTui and Schrift (14) reported that the pyrogen-producing characteristics of microorganisms depend on the type of organism, and that bacterial pyrogens are related to lipopolysaccharides.

The pyrogen test became an official quality control test for parenterals in 1942 in the U.S. Pharmacopeia (USP), 12th edition. Later, in 1945, the Code of Federal Regulations (CFR)* required antibiotics to be tested for pyrogens. Despite the advances in parenteral science and technology over the past 50 years, the rabbit pyrogen test methodology officially recognized in compendial standards has remained essentially

* CFR Title 21, Section 610.13 for biologicals and Sections 436.31 and 436.32 for antibiotics.

unchanged. The LAL test for endotoxin, discussed later, became an official USP test in 1985. Today, the LAL test, more commonly called the Bacterial Endotoxin Test, has preempted the rabbit test as the USP method of choice for detection of endotoxin in parenteral products. The traditional rabbit pyrogen test has been almost completely replaced by the more sensitive and accurate LAL assay for testing raw materials, in-process pyrogen control for pharmaceuticals and medical devices, and end-product evaluation of devices, is small and large-volume parenteral products (15). The LAL test also is widely used in the validation of depyrogenation of dry-heat sterilization processes.

SPECIFIC REQUIREMENTS OF THE USP RABBIT PYROGEN TEST

Since its inception in the USP in 1942, the rabbit pyrogen test has remained essentially unchanged. Thus, this section follows closely both the specifications written in the 22nd edition of the USP (16) and the excellent review article written in 1973 by Peroneus (17). The majority of the parenteral industry relies on the LAL test for assurance of an endotoxin free product. The discussion of rabbit testing below is included for historical data. For biologics, some countries (e.g., Canada) still require the use of the rabbit pyrogen test.

General Description of the USP Pyrogen Test

The following paragraph is quoted directly from the USP <85> under the section on pyrogen testing:

> 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 per kg injected intra-

> venously within a period of not more than 10 minutes. 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.

Apparatus and Diluents

All apparatuses—glassware, containers, syringes, needles, and soon—and all diluents used in performing the pyrogen test must themselves be free from pyrogenic contamination. Heat-durable items such as glass and stainless steel can be depyrogenated by exposure to dry heat cycles at temperatures greater than 250°C for at least 60 minutes. Diluents and solutions for washing and rinsing of devices are to be pyrogen free. Commercially available sterile and pyrogen-free solution products usually are employed.

To ensure the lack of pyrogenicity with the various materials used in conducting the pyrogen test, negative controls should be performed with each test. Negative controls utilize the diluent rather than the product sample as the injection, with the diluent being exposed to the same procedure and materials as the product sample. The use of negative controls with each pyrogen test is not standard practice because of prior knowledge and assurance that materials used in the test are nonpyrogenic.

Temperature Recording

USP <85> states the following:

> 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°C and have been tested to determine that a maximum reading is reached in less than 5 minutes. Insert the temperature-sensing probe into the rectum of the test rabbit to a depth of not less than 7.5 cm, and, after a pe-

riod of time not less than that previously determined as sufficient, record the rabbit's body temperature.

Thermocouples connected to electronic recording devices are almost exclusively used today for measuring temperature rectally in rabbits. A thermocouple contains two dissimilar electrical conductor wires joined at one end to form a measuring junction that produces a thermal electromotive force (EMF). There exist several thermocouple types, each having a defined EMF-temperature relationship. For example, at a temperature of 100°F, a type T (copper-constantan) thermocouple will generate an EMF of 1.518 mV. Common thermocouple types are listed in Table 2.2. Typical thermocouples are composed of three parts (Fig. 2.3). The two dissimilar wires are supported by an electrical insulator, either hard-fired ceramic or nonceramic materials such as Teflon, polyvinyl chloride, fiberglass, fibrous silica, or asbestos. The outer sheath can be composed of a variety of materials, most commonly stainless steel, Teflon, and various elemental metals (platinum, copper, and aluminum).

Thermocouples must be accurately calibrated against National Institute of Standards and Technology (NIST) traceable standard constant temperature baths. Accuracy of thermocouple temperature measurement can never exceed the accuracy of the thermocouple reference. Reference instrumentation should include both an ice point reference bath and an

Table 2.2 Commonly Used Thermocouple Types

Type B	Platinum–30% rhodium (+) versus platinum–6% rhodium (−)
Type E	Nickel–10% chromium (+) versus constantan (−)
Type J	Iron (+) versus constantan (−)
Type K	Nickel–10% chromium (+) versus nickel–5% (−)
Type R	Platinum–13% rhodium (+) versus platinum (−)
Type S	Platinum–10% rhodium (+) versus platinum (−)
Type T	Copper (+) versus constantan (−)

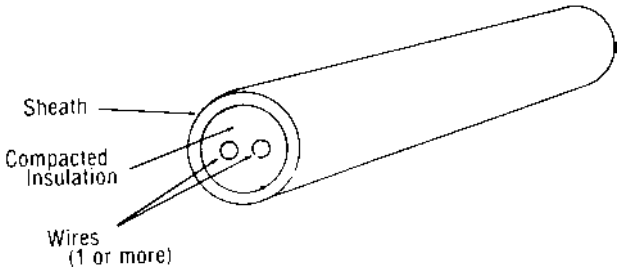

Fig. 2.3 Composition of a typical thermocouple used in rabbit pyrogen testing. (Courtesy of the American Society for Testing and Materials, Philadelphia, Pennsylvania).

elevated temperature reference bath. These calibration baths initially should be calibrated against an electronic monitor incorporating an NBS-traceable standard resistor with an accurate and constant source of current. Once the baths are calibrated, the thermocouples can be placed in the wells of the baths and temperature accuracy determined. The accuracy of the thermocouples must be $\pm 0.1°C$ of the calibration bath temperature or they should not be used in pyrogen testing.

Rabbit body temperature data are recorded electronically by instruments such as those seen in Fig. 2.4. Electronic temperature recorders usually can monitor over 100 rabbits simultaneously. Any variation in room temperature must be compensated by built-in calibration capability of the recorder. Proper maintenance and repair of recording devices must be accomplished.

Computerized equipment is now available for automatic temperature recording during pyrogen testing. A description of computerized temperature recording in pyrogen testing was published by Joubert (18).

Test Animals

Rabbits are used as pyrogen test models because they physiologically respond similarly to pyrogens as do human beings.

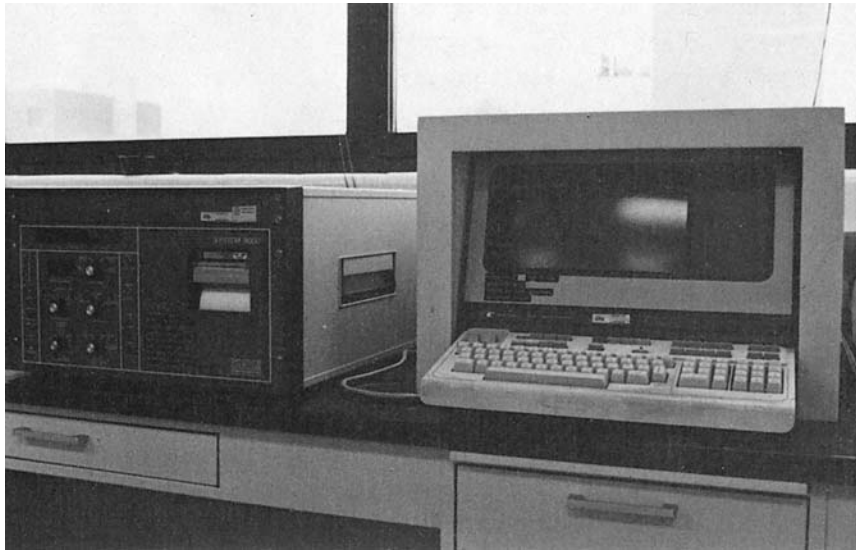

Fig. 2.4 Electronic thermal recording instruments used to monitor rabbit body temperatures during the pyrogen test.

Griesman and Hornick (19) showed that rabbits and humans respond identically on a nanogram per kilogram basis to pyrogenic quantities of endotoxin.

Quoting from the USP:

> Use healthy, mature rabbits. House the rabbits individually in an area of uniform temperature between 20°C and 23°C and free from disturbances likely to excite them. The temperature varies not more than ±3°C from the selected temperature. Before using a rabbit for the first time in a pyrogen test, condition it not more than seven days before use by a sham test that includes all of the steps as directed under Procedure except injection. Do not use a rabbit for pyrogen testing more frequently than once every 48 hours, nor prior to 2 weeks following a maximum rise of its temperature of 0.6°C or more while being

subjected to the pyrogen test or following its having been given a test specimen that was adjudged pyrogenic.

Several strains of rabbits are acceptable as test animals for the pyrogen test. Key factors in selecting rabbits are the animal breeder, rabbit resistance to disease, sufficient size for ease of handling, large ears, and rate of weight gain. The albino rabbit is the most widely used rabbit, particularly strains from New Zealand and Belgium.

It is essential that the rabbit colony be treated with utmost care. The environment in which the rabbits are housed must be strictly controlled with respect to temperature, humidity, lighting, and potential contamination of air, surfaces, and feed. Any new shipment of rabbits should be quarantined and monitored for 1 to 2 weeks following receipt of the shipment for presence of illness and/or disease.

Rabbits must be trained to adjust and adapt to their new environment in the pyrogen testing laboratory. Methods applied have been reviewed by Personeus (17). Rabbits must become accustomed to being restrained in their cages and being handled during both the rectal insertion of the thermocouple and the injection of the test product.

The normal basal body temperature of rabbits ranges between 38.9°C and 39.8°C (102.0–103.6°F). Rabbit baseline temperature is established by measuring rectal temperature during the conductance of several "sham" tests (following the entire pyrogen test procedure using pyrogen-free sodium chloride solution as the injection sample). Such tests should be, but rarely are, conducted over a period of several weeks. Temperature variances will occur in untrained rabbits, but on training, temperature variation will diminish to an acceptable range of ±0.2°C. The normal temperature range of a rabbit may shift with time, requiring the reestablishment of the true normal body temperature.

Rabbits may become tolerant to pyrogenic activity after repeated injections of endotoxin (20–22). It is for this reason

that a rabbit showing a rise of its body temperature of 0.6°C or more during a pyrogen test cannot be used again as a pyrogen test animal for at least 2 weeks.

Test Procedures

The USP procedure recommended for performing the pyrogen test is as follows:

> 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 stocks that allow the rabbits to assume a natural resting posture. Not more than 30 minutes 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 degree from each other, and do not use any rabbit having a temperature exceeding 39.8°C.
>
> 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 minutes after the 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. For exam-

ple, 40 ml of sterile, pyrogen-free saline, TS at a flow rate of approximately 10 ml per minute is passed through the tubing of each of 10 infusion assemblies. Assure that all test solutions are protected from contamination. Perform the injection after warming the test solution to a temperature of 37 ± 2°C. Record the temperature at 1 and 3 hours and 30 minute intervals in between subsequent to the injection.

Rabbits belong in a facility that is temperature controlled, for example, at 70°C ± 5°F. Housing should be individual cages designed to maintain cleanliness (see Fig. 2.5). Cage design should conform to standards established by the American Association of Accreditation of Laboratory Animal Care (AAALAC).

The facility has two basic rooms. One room houses the rabbits between tests, while the other room is used only for

Fig. 2.5 Housing of pyrogen test rabbits in clean, individual cages.

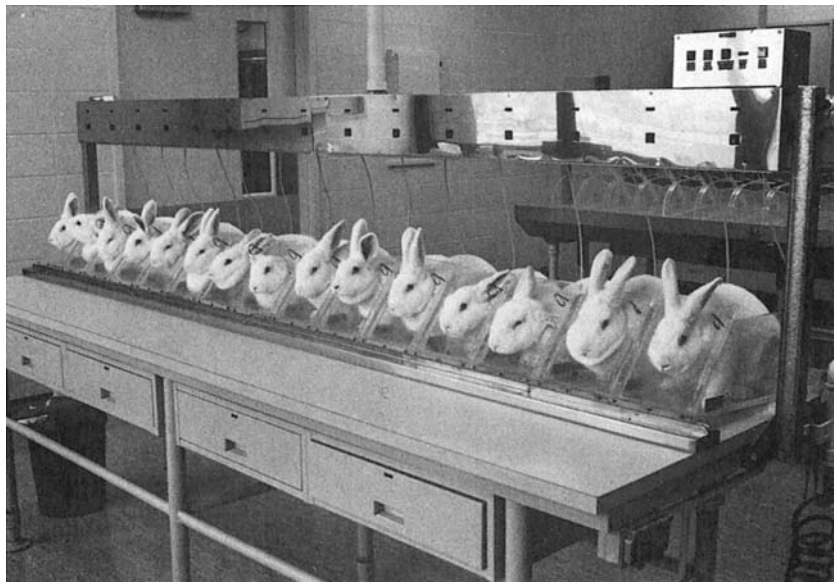

Fig. 2.6 Rabbits situated in individual restraining boxes.

actual pyrogen testing. Rabbits in restraining boxes (see Fig. 2.6) are transported on carts or wagons from the holding room into the testing room. The two rooms should have a door between them that is closed during the pyrogen testing period. Environmental conditions in the two rooms should be identical.

Noise represents a major problem in maintaining and using rabbits for pyrogen testing. The room in which the tests are conducted should be as free from noise and activity as possible. Anything that causes excitement in the rabbit potentially can produce a 0.2–1.0°C rise in body temperature that may not return to normal for 60–90 minutes.

During the pyrogen test, which could last 4 to 6 hours, the rabbits should be restrained with a minimum of discomfort. Restraint should be confined to the neck and head of the rabbit to facilitate the test dose injection into the ear vein and to permit the rabbit comfortable movement of its legs and back.

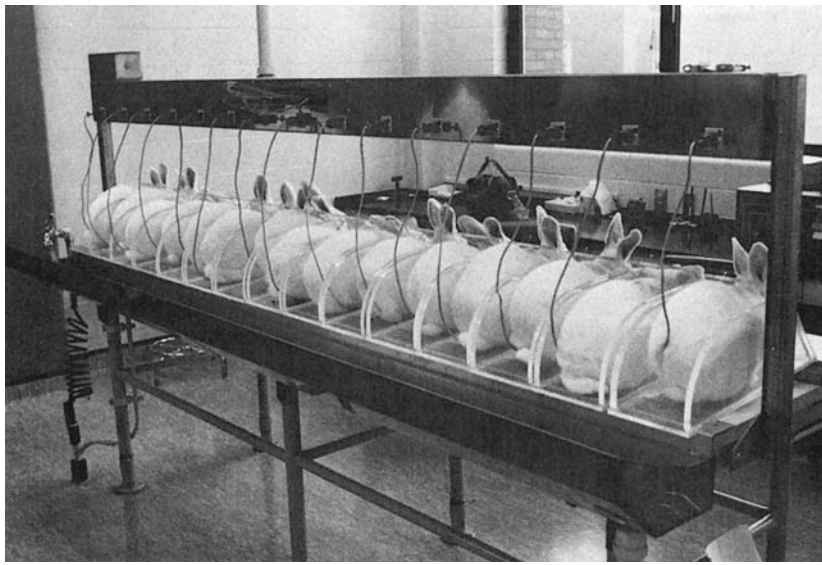

Fig. 2.7 Rear view of rabbits in restraining boxes.

Examples of modern restraining boxes are shown in Figs. 2.6 and 2.7.

Rabbits that have been adequately trained, are healthy, and exhibit stable body temperatures are selected for the pyrogen test. The animals are weighed and placed in their restraining boxes. Thermocouples (see Fig. 2.7) are inserted in the rectum to a depth of not less than 7.5 cm. Following a 30–45-minute acclimation period, the control temperature reading of the rabbit is recorded. Within 30 minutes of the recording of the control temperature, the test dose should be administered.

Dose administration is accomplished using a sterile syringe and 20–23-gauge needle. The size of syringe will depend on the dose volume. The USP requires a dose of 10 ml per kg body weight unless otherwise specified in the individual monograph. For example, for Phytonadione Injection, USP, the pyrogen test dose is 2 ml per kg, while Protamine Sulfate

Injection, USP, requires only 0.5 ml per kg containing 10 mg per ml. Some injectable monographs specify the pyrogen test dose on a weight-weight basis; for example, the dose of Diazepam Injection, USP, is 0.25 mg per kg.

The test solution must be warmed to 37°C prior to injection. The ear vein is swabbed with alcohol (70%), which not only disinfects, but also improves visibility of the vein. Vein longevity can be preserved by employing correct technique in making the injection. A suggested procedure is as follows:

1. Rest the ear against the fingers of the left hand and hold the ear down with the thumb (see Fig. 2.8).
2. Introduce the needle with the bevel edge upward near the tip of the ear vein.
3. Slowly inject a small amount of sample to determine if the needle is within the vein lumen. If not, a bubble will form or backpressure will be felt. Withdrawing the needle

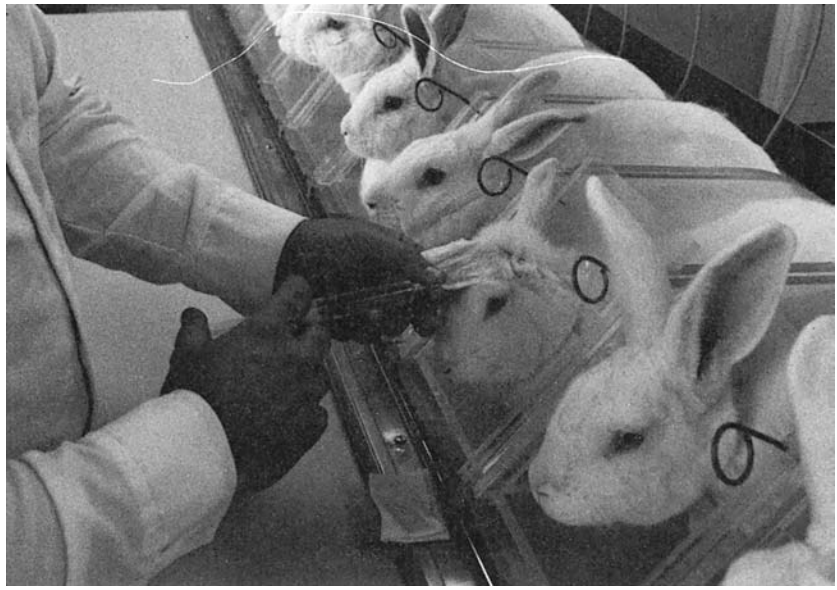

Fig. 2.8 Injection of pyrogen test sample into ear vein of rabbit.

slightly and moving it forward again should place it in proper position.
4. Maintain steady pressure on the syringe plunger and complete the injection within 10 minutes. Usually, the time duration for infusion is much less than 10 minutes.
5. Withdraw the needle and apply pressure with the thumb at the site of injection to retard bleeding and scarring.

Rectal temperatures are recorded at 1, 2, and 3 hours subsequent to the injection. During the test period, rabbits and equipment should be checked periodically. Occasionally, a rabbit may experience rectal bleeding, irritation, or leg or back discomfort. Thermocouple wires might break or the electronic thermal recorders may malfunction. Immediate action should be taken in any of these situations.

Mazur and McKendrick (23) reported on the automated pyrogen test system used by McGaw Laboratories. The system manages all phases of the pyrogen test, including setting up the test, acquiring and recording animal temperature data, calculating test results, and issuing release reports. Today, most modern pyrogen testing laboratories utilize similar computer technology.

Test Interpretation—USP

According to the July/August 1991 issue of *Pharmacopeial Forum*, the solution may be judged nonpyrogenic if no single rabbit shows a rise in temperature of 0.5°C or greater above its control temperature. If this condition is not met, the test must proceed to a second stage. There is no longer a second condition involving the sum of individual temperatures. In the second stage, five additional rabbits are given a new preparation of the same test sample as the original three rabbits. The solution may be judged nonpyrogenic if not more than three of the eight rabbits show individual temperature rises of 0.5°C or more.

The U.S. Public Health Requirements for Biological Products, Part 73, judge a solution to be pyrogenic if at least half

Table 2.3 Comparison of United States Pharmacopeial (USP) and British Pharmacopoeial (BP) Pyrogen Tests Requirements

Number of rabbits	Maximum total peak response (°C) to pass the test		Minimum total peak response (°C) to fail the test	
	USP	BP	USP	BP
3	1.4	1.15	1.4	2.65
6	—	2.80	—	4.30
8	3.7	—	3.7	—
9	—	4.45	—	5.95
12	—	6.60	—	6.60

of the rabbits tested show a temperature rise of 0.6°C or more, or if the average temperature rise of all rabbits is 0.5°C or more.

The British Pharmacopoeia (BP) (24) pyrogen test employs a sliding scale based on 3 rabbits and additional groups of 3 rabbits, if required, for a total of 12 rabbits. This scale is shown in Table 2.3 with the former USP test included for comparison.

Limitations of the USP Rabbit Pyrogen Test

The USP rabbit pyrogen test suffers from several limitations, which established the opportunity for the *Limulus* Amebocyte Lysate test as a possible alternative for the rabbit test as an official pyrogen test procedure.

In Vivo Model

A test method (the in vivo model) that uses a living animal as its model certainly must submit to a number of problems offered by biological systems. Variability in biological systems poses a great problem. No two rabbits will possess exactly the same body temperature or respond identically to the same pyrogenic sample. Rabbits are extremely sensitive and vulnerable to their environment. This translates into an expensive

proposition in terms of facilities, control of the environment, and training of the animal.

Pyrogen testing of rabbits is not only expensive, but also laborious. Several hours are consumed in performing the pyrogen test, including a great amount of preliminary effort in preparing the animals. Rabbits must be fed and watered properly, cages cleaned to prevent disease, and time spent in training the animals to adapt to the conditions of the pyrogen testing facility and the test itself.

Rabbit Sensitivity to Pyrogens

The pyrogenic response in rabbits is dose dependent. The greater the amount of pyrogen injected per kilogram body weight, the greater the temperature increase in rabbits. This is demonstrated in Table 2.4, taken from a report by Mascoli and Weary (25).

A collaborative study initiated under the auspices of the Health Industry Manufacturers Association (HIMA) demonstrated that rabbits from 12 laboratories consistently failed the (pyrogenic) test at doses of 1.0 ng per ml (10 ml/kg of 10 ng/kg endotoxin) of *Escherichia coli* 055:B5 endotoxin, and all colonies passed (no pyrogenicity) at the 0.156 ng/kg dose (or 0.156 ng/ml using a 10 ml/kg dose) (25). The same study reported that the "average" rabbit colony will attain a 50% pass/fail rate with 95% confidence at an endotoxin level above 0.098 ng/ml (10 ml/kg dose). The LAL test generally will detect endotoxin levels of 0.025 ng/ml or less. Thus, the rabbit test is less sensitive to endotoxin than the LAL test is.

Rabbit-to-rabbit variation in response to the same lot of pyrogenic solution was shown by Mascoli and Weary (25). As seen in Table 2.4, the standard deviations and coefficient of variation values are rather high among eight rabbits administered identical doses of endotoxin. The HIMA study reported that, of 12 laboratories conducting rabbit pyrogen tests, 4 passed a level of 2.5 ng endotoxin per kg (26).

Sensitivity of the rabbit bioassay for endotoxin appears to fall in the range of 1 to 10 ng/kg (19,27). Greisman and

Table 2.4 Eight Rabbit Pyrogen Test Results in Saline with *Escherichia coli* 055:BS Using 3–5 kg Rabbits

E. coli endotoxin concentration (ng/ml)	Volume solution injected (ml/kg)	USP total temperature increase (°C)	Mean temperature increase (°C)[a]	Standard deviation (°C)[b]	Coefficient of variation (%)
3.125	1.0	7.80[c]	0.975	0.246	25.2
1.56	1.0	4.75[c]	0.594	0.218	36.7
1.00	1.0	3.70[c]	0.462	0.158	34.2
0.78	1.0	1.40	0.144	0.208	144.4
0.39	1.0	1.00	0.088	0.187	212.5
0.195	1.0	1.20	0.150	0.065	43.3

[a] Negative rabbit temperature values were excluded from total temperature increase determinations according to USP.
[b] Negative rabbit temperature values were included in the determinations of means and standard deviations to properly reflect total variability.
[c] Failed USP test criteria of 3.7°C total increase.
Source: Ref. 25.

Hornick (19) found that the threshold pyrogenic dose of *E. coli* endotoxin for both rabbits and humans is 1.0 ng/kg of body weight. This holds true regardless of the volume of pyrogenic solution administered because of the dose (rather than concentration) dependency of the rabbit response to pyrogen.

Rabbit sensitivity to endotoxin varies with the time of day (circadian) and time of year (cirannual) (28). The greatest rise in temperature for any given dose of endotoxin occurred in the afternoon, while the least rise occurred at midnight. At midnight, the greatest sensitivity was seen at the end of October, while the least was seen at the end of April. However, this was opposite at 10:00 A.M. Although not practical at all, it was suggested in this report that a rabbit colony be tested for its threshold sensitivity at the beginning of each month and at the hours when products would be tested normally. Thus, seasonal variability in sensitivity may be controlled.

Interferences of the Rabbit Pyrogen Test

Many products administered parentally cannot be tested for pyrogens with the rabbit test because of the interferences they create in the rabbit response to pyrogens if they are present in the product. Any product having a pyretic side effect, such as the prostaglandins and the cancer chemotherapeutic agents, will interfere with the rabbit response. Several products are inherently toxic to the rabbit (see Table 2.5) and must

Table 2.5 Examples of Drugs and Drug Products Not Suitable for Testing by the USP Pyrogen Test

Most cancer chemotherapeutic agents
Most anesthetics, muscle relaxants, and sedatives
Sterile betamethasone sodium phosphate solution
Chlorpheniramine injection
Magnesium sulfate
Metocurine iodide injection
Perphenazine
Thiopental sodium for injection

be diluted to concentrations far below the pharmacologically effective dose of the drug.

Despite these major limitations and the insurgence today of the LAL test, it must not be forgotten that the USP rabbit pyrogen test for decades has nobly served as a sufficiently sensitive test for pyrogens and has helped to eliminate pyrogenic contamination from drugs reaching the marketplace, although most pharmaceutical and device manufacturers currently use the LAL test for the pyrogen test.

The official "referee" test according to the USP and EP is the LAL gel clot test.

THE *LIMULUS* AMEBOCYTE LYSATE TEST

History and Background

Credit for discovering the interaction between endotoxin and the amebocyte lysate of the horseshoe crab, *Limulus polyphemus*, belongs to Levin and Bang (29). Basing their work on earlier research by Bang (30), these workers were involved in the study of clotting mechanisms of the blood of lobsters, fish, and crabs. Autopsies of dead horseshoe crabs revealed intravascular coagulation. The clotted blood was cultured and found to contain gram-negative bacteria such as *E. coli* and *Pseudomonas*. Further tests showed that amebocyte cells of the blood of the horseshoe crab blood were extremely sensitive to the presence of endotoxin, the toxic substance liberated by the disintegration of bacterial cells. The substance in the amebocytes responsible for reacting with endotoxin is known to be a clottable protein, discussed in the section, "Reaction Mechanism." In lysing the amebocyte cells by osmotic effects, a most sensitive biochemical indicator of the presence of endotoxin was produced, hence the name *Limulus* Amebocyte Lysate test.

Limulus polyphemus (see Fig. 2.9) is found only at specific locations along the East Coast of North America and the coasts along Southeast Asia. The hearts of mature crabs are punctured and bled to collect the circulating amebocyte blood

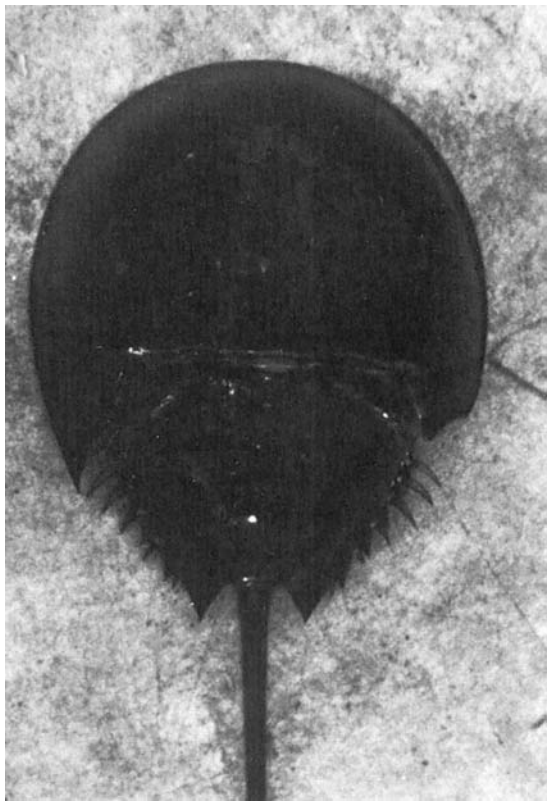

Fig. 2.9 *Limulus polyphemus*, the source of *Limulus* amebocyte lysate reagent.

cells. Carefully performed, this procedure is not fatal to the crab, and on proper restoration, the crab can be used again. Since amebocytes act as activators of the coagulation mechanism in the crab, an antiaggregating agent must be added to inhibit aggregation. N-Ethylmaleimade is the most commonly used anti-aggregant.

Amebocyte cells are collected and washed by centrifugation and lysed using distilled water. Lysing can also be done with ultrasound, freezing and thawing, and grinding in a glass tissue homogenizer (31). After lysing, the suspension is

cleared of debris by centrifugation, and the supernate is lyophilized. Lyophilization is necessary for stability purposes. LAL reagent is extremely sensitive to heat, and even in the lyophilized state must be stored in the freezer (32). On reconstitution, LAL has a shelf life of 1 month storage at freezing conditions.

The LAL test for pyrogens in parenterals was first applied by Cooper et al. (33). The LAL test was found to be more sensitive and, certainly, more expedient than the rabbit pyrogen test in the testing of radioactive drug products. Mallinckrodt, Incorporated, established the first successful, large-scale production facility for LAL in Chincoteague, Virginia, in 1971 (34).

On January 12, 1973 (*Federal Register 38*, 1404), the Food and Drug Administration (FDA) stated that LAL was a biological product and thus was subject to licensing under Section 351 of the Public Health Service Act. Specifications concerning the purity and potency of LAL were proposed by the FDA Bureau of Biologics (now CBER) later that year (September 18, 1973; 38 FR 26130). In the ensuing years, available data on and experience with the LAL test accumulated, with the primary use of the test being an in-process endotoxin test. Finally, the FDA announced conditions under which the LAL test could be used as an end-product test for licensed biological products and medical devices (November 4, 1977; 42 FR 57749). This was followed by a draft guideline published by the Office of Medical Devices for using the LAL test for medical devices exclusively (March 20, 1979).

In the *Federal Register* of January 18, 1980 (45 FR 3668), the FDA published a notice announcing the availability of a draft guideline describing the conditions for validating the LAL test before using it as a final end-product endotoxin test for human and veterinary injectable drug products. Comments on the two draft guidelines (March 1979 and January 1980) resulted in a single draft guideline for validation of the LAL test as an end-product endotoxin test for human and animal parenteral drugs, biological products, and medical de-

vices; this was published on February 2, 1983, and announced on March 29, 1983 (48 FR 13096).* Specific details of this guideline are identified in later sections of this chapter.

Until 1977, the Bureau of Biologics prepared its own lysate. Since then, the Bureau has found it more economical to purchase licensed lysate from one of several licensed manufacturers. The specifications required by the FDA before purchasing a lot of LAL are summarized in Table 2.6 (36).

Reaction Mechanism

Elucidation of the endotoxin-LAL reaction has resulted primarily from the work by Liu et al. (37), Takagi et al. (38), and Mosesson et al. (39). Combining the results of these researchers' efforts produces the following proposed reaction:

1. Endotoxin or a suitably prepared lipid A derivative of endotoxin activates a proenzyme of LAL having a molecular weight of 150,000.
2. Activation also depends on the presence of divalent metal cations such as calcium, manganese, or magnesium. It has been shown that the sensitivity of the LAL assay for endotoxin detection can be increased 10 to 30 times by using LAL reagent containing 50 mM magnesium (40).
3. The activated proenzyme, related to the serine protease class containing such enzymes as thrombin, trypsin, and factor Xa, subsequently reacts with a lower molecular weight protein fraction (MW = 19,000–25,000) contained also in the LAL substance.
4. The lower molecular weight fraction, called coagulogen, is cleaved by the proenzyme into a soluble and insoluble subunit. The insoluble subunit appears as a solid clot, a precipitate, or a turbid solution, depending on the amount of insoluble coagulogen by-product formed.

* In December 1987, the FDA published its final guideline on validation of the LAL test as an end-product test for endotoxin for all products (human and animal parenteral products, biological products, and medical devices) (35).

Table 2.6 Summary of FDA Standards Governing the Manufacture of *Limulus* Amebocyte Lysate Reagent

Use of U.S. Standard Endotoxin for determining the sensitivity of LAL.
Use of U.S. Reference LAL for establishing the potency of LAL.
Calculation of potency of each lot of LAL and the U.S. Reference LAL using the U.S. Standard Endotoxin.
a. Test a minimum of 20 to a maximum of 28 vials per each drying chamber.
b. The 99% fiducial upper limit of the standard deviation of the log ratio of reference and test lysates for 20 vials can be no greater than 0.73.
General requirements.
a. Handle horseshoe crabs in a manner to enable them to be returned alive to their natural environment after a single collection of blood.
b. Perform sterility test on bulk lot and on each filling.
c. Run negative control tests of lysate.
d. Test for residual moisture.
Various labeling requirements.
Appropriate number of samples (not fewer than 28 vials) and documentation of manufacture of each filling, dates of testing, and results of all tests must be submitted to Director, Bureau of Biologics, FDA.

Source: Ref. 36.

Therefore, the coagulation reaction requires three factors in addition to endotoxin. These three factors—a clotting enzyme, clottable protein (coagulogen), and certain divalent cations—are found in the LAL reagent. A schematic representation of the LAL reaction mechanism is found in Fig. 2.10 (41).

LAL Test Procedure

Cooper (42) first described the methods and materials required to perform correctly the LAL test for pyrogen. While the LAL test is a relatively simple procedure, especially when

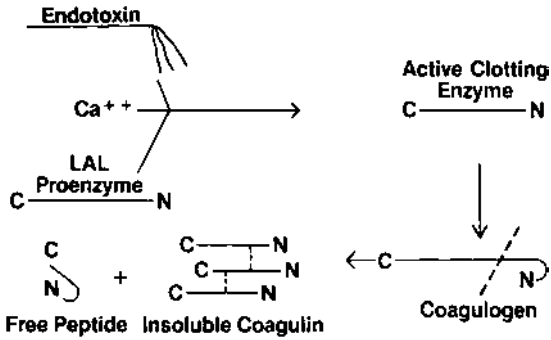

Fig. 2.10 Schematic representation of the LAL reaction mechanism. (From Ref. 41).

compared with the USP rabbit test, certain specific conditions must be met. These include:

1. All materials that will come into contact with the LAL reagent or test sample must be thoroughly cleaned and depyrogenated.
2. The reaction temperature cannot be outside the range 36–38°C.
3. The reaction mixture must be within the range of pH 5–7.
4. The reaction time should be no longer than 1 hour.
5. Each test must be accompanied by positive and negative controls.

The basic procedure of the LAL test is the combination of 0.1 ml test sample with 0.1 ml LAL reagent. After 1 hour incubation at 37°C, the mixture is analyzed for the presence of a gel clot. The LAL test is positive, indicating the presence of endotoxin, if the gel clot maintains its integrity after slow inversion of the test tube containing the mixture (see Fig. 2.11).

Complete instructions for conducting the LAL test are found in inserts supplied with LAL test kits from commercial manufacturers. The USP (22nd edition) also contains instructions for using the LAL test to estimate the concentration of

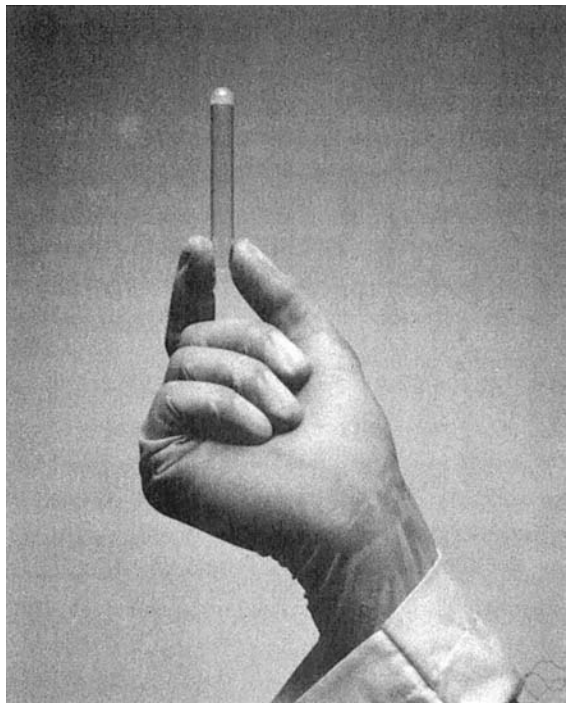

Fig. 2.11 A positive LAL gel clot test is characterized by the formation of a solid gel that remains intact in the bottom of the tube on inversion. (Courtesy of BioWhittaker, Inc., Walkersville, Maryland).

bacterial endotoxins in sample materials. These instructions are summarized with commentary below:

Preliminary

1. Strict aseptic technique must be used to avoid microbial contamination while conducting the test.
2. All containers and equipment used must be pyrogen free. Heating at 250°C or above for at least 60 minutes should depyrogenate these items.
3. All glassware should be washed with detergent prior to dry heat depyrogenation. If detergent is not completely

rinsed, it will interfere with the reaction and cause a false-negative result.

4. Abide by all precautions in reconstituting and storing the test reagents. Do not store diluted endotoxin used to determine LAL sensitivity because of loss of activity by adsorption to glass surfaces. The normal shelf life for LAL reagent is 4 weeks at freezing temperatures after reconstitution.

Standards

For drugs, biological products, and medical devices, the endotoxin standard is called the U.S. Standard Endotoxin or the USP Reference Standard Endotoxin (RSE). The first RSE lot was designated as Lot EC-2 and had a defined activity of 1 endotoxin unit (EU)* in 0.2 ng of the standard (43). The current FDA and USP reference standard is purified lipopolysaccharide from *E. coli* 0113. One vial contains 10,000 EU.

When the USP selected the FDA endotoxin standard (purified lipopolysaccharide from *E. coli* 0113) as the new USP reference standard (with established potency in endotoxin units), this gave manufacturers the opportunity to standardize their own control standard endotoxin (CSE) against the USP RSE.

There are three LAL manufacturers licensed by the U.S. government (44). Each manufacturer must determine the sensitivity of each lot by using the U.S. Standard Endotoxin EC-5, which is identical to the USP Endotoxin Standard (Lot F). The FDA tests each lot for potency before releasing it to be marketed.

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* It has become accepted practice to use endotoxin units as the more desirable expression of endotoxin strength than weight or concentration terms. The use of endotoxin units will allow any endotoxin type or lot to be used as a reference lot because its activity can always be related to the original U.S. Reference Standard lot. This chapter uses the endotoxin unit term as much as possible, but most literature references cited use the weight or concentration terms as reported in the published articles. It is noted in USP XXV and the 2002 EP that 1 EU = 1 IU (international unit).

If a manufacturer chooses to use an endotoxin preparation (CSE) other than the U.S. RSE, the CSE will have to be standardized against the RSE. What this means is that the CSE reaction in the rabbit, its uniformity, its stability, and its interaction to a particular LAL lot all must be determined and related to these same characteristics of the RSE.

1. At least four vials of the lot of CSE should be assayed by determining end points (gelations) with LAL. The values obtained should be the geometric mean of the end points using a minimum of four replicates.
2. The end point for the CSE is stated in nanograms per milliliter. The end point for the RSE is endotoxin units per milliliter. So, if the LAL end point for the CSE is 0.018 ng/ml and the LAL end point for the RSE is 0.3 EU/ml, then

$$\frac{\text{RSE} = 0.3 \text{ EU/ml}}{\text{CSE } 0.018 \text{ ng/ml}} = 16.6 \text{ EU/ng of CSE}$$

3. This indicates that 0.018 ng of the CSE is equal to 0.3 EU of the RSE. Thus, the CSE contains 16.6 EU/ng.

Validation of the LAL Test

To validate the use of the LAL test for any application requires two determinations: initial qualification of the laboratory and inhibition or enhancement properties of the product on the LAL-endotoxin interaction. Extensive details of LAL test validation requirements are found in the "Guideline on Validation of the *Limulus* Amebocyte Lysate Test as an End-Product Endotoxin Test for Human and Animal Parenteral Drugs, Biological Products, and Medical Devices" (35).

Qualification of the laboratory simply involves using the selected test method (gel clot end point, chromogenic and end point–turbidimetric, or kinetic-turbidimetric techniques) to determine its variability, to test new lots of lysate before use, and to qualify the ability of the analyst(s) to conduct the test. The LAL reagent used must have a confirmed potency (sensi-

tivity). This is achieved by combining the particular reagent with a series of concentrations of RSE or CSE endotoxin, bracketing the stated sensitivity (EU/ml) of the LAL reagent. Use four replicates per concentration of endotoxin. The series of endotoxin concentrations is prepared by twofold dilutions of the RSE or CSE endotoxin using LAL-negative water for injection. Following incubation and end-point determination (manual or instrumental), the sensitivity of the LAL reagent will be confirmed if the test results are positive to within one twofold dilution of the stated label potency.

Inhibition/enhancement testing must be performed on undiluted drug products or diluted drug products not exceeding the maximum valid dilution value (see Table 2.7) (35). At least three production batches of each finished product should be tested. The product is spiked with various known amounts of RSE (or CSE), bracketing the sensitivity of the lysate used, using four replicate reaction tubes per level of endotoxin. The same number of tubes is used for drug product containing no added endotoxin and for control water for injection samples also spiked with various known amounts of RSE or CSE. The LAL test procedure is carried out manually or instrumentally.* The end points (E in units per ml) are then observed and recorded for all replicate samples.

The end points are determined followed by computation of the geometric mean of these end points. Geometric mean is

$$\frac{\sum \text{E (end points)}}{\text{f (number of replicates)}}$$

and this mean is calculated for the control and test samples. An illustration is given in Table 2.8 (41). The geometric means of the product sample and the water control sample are compared. If the product sample mean is within twofold of the con-

* The FDA validation guideline contains specific directions for inhibition/enhancement testing depending on the technique used—gel clot, inorganic and end point–turbidimetric, and kinetic turbidimetric.

Table 2.7 Examples of Minimum Valid Concentration (MVC) and Minimum Valid Dilution (MVD) Calculations

MVC determination

$$MVC = \lambda M/K$$

λ = Sensitivity of LAL reagent in endotoxin units per milliliter
M = Rabbit dose or maximum human dose per kilogram
K = 5.0 E/kg (0.2 EU/kg for intrathecal drugs)

If LAL sensitivity (λ) was 0.065 EU/ml and the maximum human dose were 25 mg/kg, then the MVC would be

$$MVC = \frac{0.065\text{EU/ml} \times 25\text{ mg/kg}}{5.0\text{ EU/kg}} = 0.325\text{ mg/ml}$$

If this dose were to be given intrathecally, the denominator would be 0.2 EU/kg.

MVD determination

$$MVD = \frac{\text{Potency of product}}{MVC} = 1{:}61.5$$

If the potency of a product were 20 mg/ml, the MVD would be

$$MVD = \frac{20\text{ mg/ml}}{0.325\text{ mg/ml}} = 1{:}61.5$$

Therefore, this product can be diluted to 61.5 times its original volume and still be able to detect the lower endotoxin concentration limit by the LAL test.

Source: Ref. 35.

trol mean sample, the drug product is judged not to inhibit or enhance the LAL-endotoxin reaction. For example, if the product sample showed a geometric mean of 0.4 EU/ml and the water control mean was 0.2 EU/ml, the LAL test is valid for that product.

If endotoxin is detectable in the untreated specimens under the conditions of the test, the product is unsuitable for the inhibition/enhancement test. Either endotoxin must be removed by ultrafiltration or further dilution can be made as

Table 2.8 Example of Geometric Mean Determination for a Small-Volume Parenteral Product Undergoing LAL Testing for Endotoxin[a]

Replicates (f)	Unit y	Gel end-point results for specimen dilutions 0.5	0.25	0.125	End-point dilution factors (E)
1	+	+	+	−	0.25
2	+	+	−	−	0.5
3	+	+	−	−	0.5
4	+	+	+	−	0.25
5	+	+	−	−	0.5
					$\sum E = 2.0$

[a] Geometric mean = $\sum E/f = 2.0/5 = 0.4$.
Source: Ref. 41.

long as the minimum valid dilution (MVD) is not exceeded, and the inhibition/enhancement test repeated. If the drug product is found to cause inhibition or enhancement of the LAL test, the following courses of action can be taken (35):

1. If the drug product is amenable to rabbit testing, then the rabbit test will still be the appropriate pyrogen test for that drug.
2. If the interfering substances can be neutralized without affecting the sensitivity of the test or if the LAL test is more sensitive than the rabbit pyrogen test, then the LAL test can still be used.
3. For those drugs not amenable to rabbit pyrogen testing, the manufacturer should demonstrate that the LAL test can detect the endotoxin limit established for the particular drug. If the limit cannot be met, the smallest quantity of endotoxin that can be detected must be determined.

There are various miscellaneous requirements in the procedures for validating the LAL test:

1. Use positive and negative controls in all tests.
2. Use the highest and lowest drug concentrations for drug products marketed in three or more concentrations.
3. Use three lots of each drug concentration for the validation tests.
4. If the lysate manufacturer is changed, the validation test must be repeated on at least one unit of product.
5. The LAL reagent should have a sensitivity of at least 0.25 EU/ml.
6. The endotoxin control must always be referenced to the RSE.
7. Any change in the product formulation, manufacturing process, source of formulation ingredients, or lot of lysate necessitates a revalidation of the LAL test for the product.

The possibility of a device inhibiting or enhancing the LAL-endotoxin reaction is determined by extraction testing of each of three device production lots. The extract solution must be pyrogen-free water or saline to which known amounts of standard endotoxin, bracketing the sensitivity of the lysate, have been added. Depending on the type of device, extracts may be obtained by flushing, immersing, or disassembling, then immersing the device with the endotoxin-spiked solution. The LAL test results of the extract should not be different from the results of testing standard solutions containing endotoxin that have not been exposed to the device.

Endotoxin highly adsorbs to container surfaces. Novitsky et al. (45) reported on the different adsorptive natures of container surfaces. Recovery of endotoxin occurred with polystyrene containers, while the worst for recovering endotoxin were polypropylene containers. In fact, regardless of extraction method, less than 1% endotoxin was ever recovered from polypropylene containers. Bonosilicate glass allowed higher recovery than flint glass.

Great care must be exercised in preparation and storage of parenteral vials used for LAL testing. Guilfoyle et al. (46) reported that 20–40% of spiked endotoxin in vials was lost due

to adsorption to rubber stoppers. The authors suggested that product containers be stored in an upright position and a uniform mixing procedure prior to assay be established.

Manual LAL Test Procedure

Four or more replicate samples at each level of the dilution series for the test samples are used in most cases. The pH of the reaction mixture must be between 6.0 and 7.5 unless specified differently in the particular monograph. The pH may be adjusted by addition of sterile, endotoxin-free 0.1 N sodium hydroxide, 0.1 N hydrochloric acid, or suitable buffers.

Test tubes, usually 10 by 75 mm, are filled with an aliquot, usually 0.1 ml, of reconstituted LAL reagent and the same aliquot volume of the test sample. In other test tubes, equal volumes of LAL reagent and endotoxin standard are combined. Positive controls (LAL reagent sample containing a known concentration of endotoxin) and negative controls (LAL reagent plus an equal volume of sterile, pyrogen-free solvent) are run simultaneously with the test samples and endotoxin standards.

When the equal volumes are combined, the test tube is swirled gently. The tube is placed in a constant temperature water bath with temperature controlled at 37°C ± 1°C. Incubation times ideally last 60 ± 2 minutes. While incubating, the test tubes must never be disturbed for fear of irreversibly disengaging the gel clot if it has formed. Careful removal of the incubated test tubes for gel clot analysis is extremely important.

The use of microscope slides containing petrolatum wells has been advocated for conducting the LAL test when lower reagent consumption is desired (47). One slide can accommodate 12 samples using microliter volumes (0.1 μl). A dye solution (0.1% toluidine blue in ethanol) is placed in each well to aid in interpreting the results. A positive LAL test generates a blue "star" in the droplet, while a negative LAL test gives a homogeneous blue solution.

The degree of gel formation can be determined by either

direct visual observation or instrumental analysis. Visual observation starts by carefully removing the test tube from the incubator, then carefully inverting (by 180°) the test tube and visually checking for the appearance of a firm gel. A positive reaction is characterized by the formation of a firm gel that does not break or lose its integrity during and at the completion of the inversion process. A negative result is characterized by the absence of a gel or by formation of a viscous gel that does not maintain its integrity during the inversion process. An example of a positive LAL test result is seen in Fig. 2.11.

Instrumental Tests

Direct visual observation of the gel end point relies on the subjective interpretation of the observer and, unless twofold serial dilutions are performed, provides only a qualitative (yes or no) measurement of the endotoxin present in the sample. Analysis of the gel end point by instrumental methods offers several advantages, including single-tube quantitation and objectivity. In addition, instrumental methods can be automated, resulting in increased speed, efficiency, and adaptation to computer control.

Two basic instrumental methods are available for LAL testing. One method is based on turbidimetric measurement of gel formation (e.g., Abbott's MS-2, Millipore's Pyrostat), while the other method is based on colorimetrically measuring a chromophobic substance produced during the LAL-endotoxin reaction (e.g., Mallinckrodt and BioWhittaker).

The Abbott MS-2 Microbiology System was designed originally for automated antibiotic susceptibility testing of clinical samples. The system was first described by Jorgensen and Alexander (48) and later by Novitsky et al. (49). A general procedure is outlined below:

1. LAL is mixed with the test sample in a 1:4 ratio, (e.g., 100 µl LAL + 400 µl test) in a polystyrene research cuvette.
2. Up to 88 samples can be incubated per module. Incubation occurs at 35°C for 60 minutes.
3. The mixture of each cuvette following incubation is exam-

ined for turbidity (light transmission) by recording the optical density (OD) at 670 nm on the MS-2 spectrophotometer. The samples are examined at either 1- or 5-minute intervals.

4. The OD values are recorded on a cassette tape and/or paper and can be displayed graphically as OD versus time on a cathode ray tube or transferred to paper with a hardcopy printer. An example of a plot of OD at 670 nm versus time using standard endotoxin samples is shown in Fig. 2.12 (49).

Turbidimetric OD has been shown to be directly proportional to *E. coli* endotoxin concentration on a log-log plot. For example, Fig. 2.13 shows such a relationship. Standard curves usually are linear only within a relatively small concentration range, for example, 0.01–0.1 ng/ml (0.1–1.0 EU/ml). The establishment of standard curves for instrumental analyses of the LAL-endotoxin reaction can be difficult. The availability of standard endotoxin has improved the reproducibility of standard curve determinations.

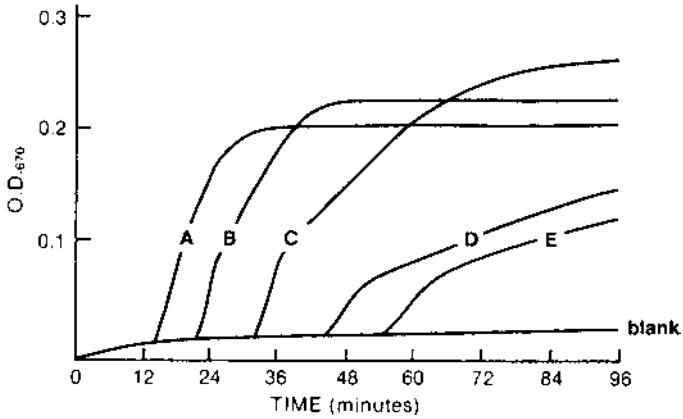

Fig. 2.12 Turbidimetric response of LAL with control standard endotoxin diluted in sterile water for irrigation. A, 100 pg endotoxin/nl; B, 2.5 pg/nl; C, 6.3 pg/nl; D, 1.6 pg/nl; E, 0.4 pg/nl. (From Ref. 49).

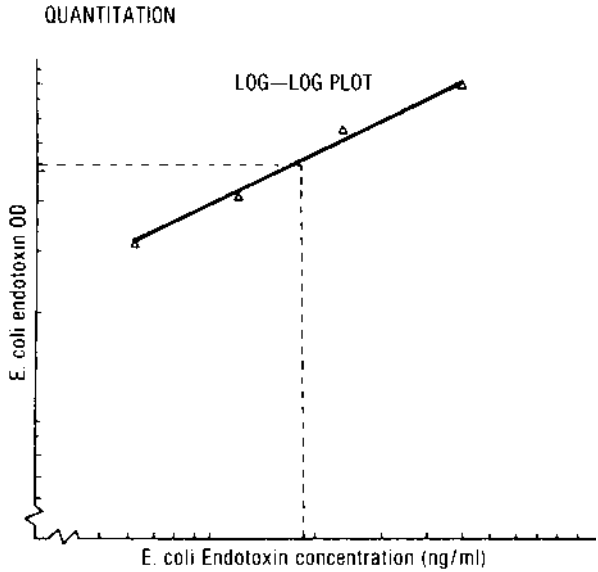

Fig. 2.13 Log-log relationship between turbidimetric optical density and endotoxin concentration. (Courtesy of Millipore Corp., Bedford, Massachusetts).

Another aspect of the kinetic LAL test that presents challenges for the parenteral manufacturer is CSE spike recovery. According to USP and EP, the CSE spike recovery in product must be 50–200%. Findings by Zink-McCullough et al. have shown that shown that CSE can be recovered in a battery of products by dilution (154). A practical example of the use of the Abbott MS-2 automated LAL test system in the detection of bacteriuria was published by Jorgensen and Alexander (50). The use of turbidimetry in automated LAL testing provided a way of successfully analyzing endotoxin in blood (51). Automated microliter testing overcomes the inhibitory factors in blood that mask the gelatin reaction using conventional LAL test methodology.

A newer type of automated LAL test system is based on the measurement of color intensity of the LAL gel end point.

This system is called the Chromogenic LAL assay system (Fig. 2.14). The test sample is mixed with LAL reagent and incubated at 37°C for a period of time (usually 10 minutes). A substrate solution containing a color-producing substance is then mixed with the LAL test sample and incubated at 37°C for an additional 3 minutes. The reaction is stopped with 50% acetic acid. The color absorbency of the sample mixture is determined spectrophotometrically at 405 nm. The more intense the color, the greater the absorbance value measured. Endo-

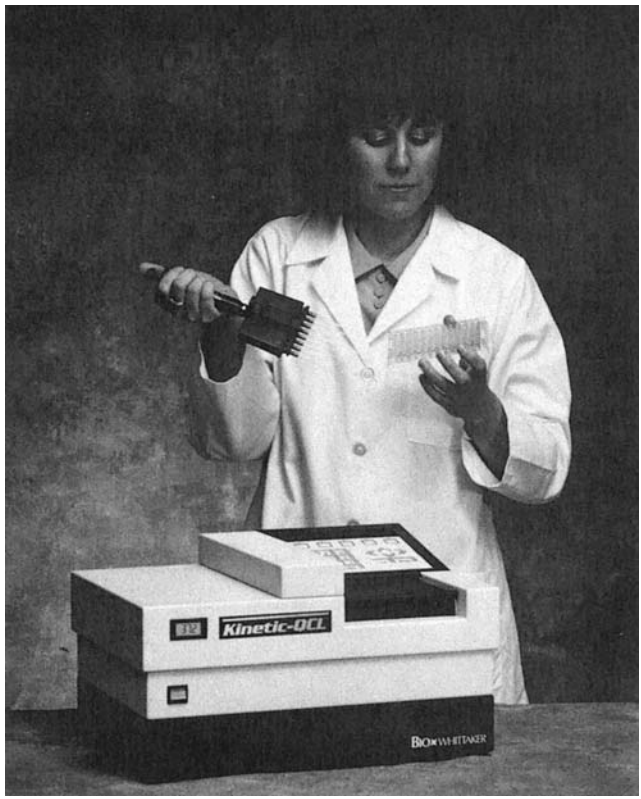

Fig. 2.14 An example of automated LAL technology is the kinetic chromogenic method, one instrument of which is shown here. (Courtesy of BioWhittaker, Inc., Walkersville, Maryland).

toxin concentration can then be determined from a standard plot of absorbance versus endotoxin concentration in nanograms per milliliter or endotoxin units per milliliter.

The chemical composition of the substrate is a peptide chain linked to p-nitroaniline (pNA) (52). The endotoxin catalyzes the activation of a proenzyme in the LAL, as discussed on pages 124–125. The activated enzyme, in turn, catalyzes the splitting of pNA from the colorless substrate. In Fig. 2.10, pNA replaces coagulogen as the substance cleaved by the proenzyme. It is pNA that is measured spectrophotometrically. Absorbance at 405 nm and endotoxin concentration are linearly related between 0.01 and 0.1 ng/ml.

For laboratories responsible for conducting multiple LAL tests, automation practically becomes a necessity. Automation employs all the advantages of instrumental analyses, including greater precision and sensitivity. Technology has advanced to the point at which the LAL test can be performed automatically using robotic systems such as one produced by Zymate (53). Such a system will automatically dilute a stock reference endotoxin standard for construction of a five-point standard curve, make sample dilutions to the proper testing concentration, and perform chromogenic substrate LAL assays in duplicate. In 48 minutes, the automated system assays three samples and a reference standard in duplicate along with a water blank. The method can be sensitive to a detection limit of 0.003 EU/ml with 30 minutes of incubation. Assay precision is approximately 6%. The major disadvantages of automated LAL testing systems are their cost and complexity. Cooper (54) recommended that each laboratory carefully consider its present and long-term needs and be firmly grounded in the fundamentals of the LAL test before changing from manual to automated LAL test systems.

Lindsay et al. (55) described a new reagent for the chromogenic LAL assay. A single reagent now contains the LAL components, buffer, and the chromogenic substrate.

The LAL test requirements for lack of pyrogenicity or

critical endotoxin concentration will be met if there is no formation of a firm gel at the level of endotoxin specified in the individual monograph. For instances when instrumental analyses have been done, the sample will pass the LAL test if not more than the maximum permissible amount of endotoxin specified in the individual monograph is present in the sample. In addition, the confidence limits of the assay must not exceed the limits previously specified for the instrumental analysis.

Endotoxin Limits in Parenteral Articles

Endotoxin limits are necessary because bacterial endotoxin is ubiquitous and is expected to be present in all articles at some level. The question is, What level is safe? This becomes the endotoxin limit (57).

The first FDA draft guideline for LAL testing of drugs (58) proposed an endotoxin limit for all parenterals of 0.25 EU/ml. This limit was vehemently opposed by the parenteral drug industry because the limit was arbitrary, based on concentration rather than endotoxin quantity per dose, and did not permit sufficient dilution of small-volume parenterals known to inhibit the LAL test reaction.

The Parenteral Drug Association proposed an alternative endotoxin limit based on rabbit or human dose (59); the FDA accepted this alternative and it became part of the new FDA draft guideline for end-product testing published in December 1987 (60). The new endotoxin limit is:

$$\frac{\text{K} = \text{Threshold Pyrogen Dose (TDP)}}{\text{M} = \text{Maximum rabbit or human dose}}$$

where the TPD has been defined as 5 EU/kg, the lower 95% confidence limit of the average dose found to produce a pyrogenic response in rabbits and humans (61). For drugs administered intrathecally, for which pyrogenic contamination can be much more dangerous (see pp. 154–155), the TPD is 0.2 EU/kg.

The maximum rabbit or human dose is that dose administered per kilogram of body weight of rabbit or human in a period of a single hour, whichever is larger. For example, if a drug with a concentration of 1 mg/ml has a maximum human loading of 25 mg/kg while the rabbit pyrogen test dose is 10 mg/kg, the maximum dose used in the denominator of the endotoxin limit equation would be the human dose of 25 mg. On the other hand, were the above human dose only 2.5 mg/kg, then the rabbit dose of 10 mg/kg would be the larger of the two doses. The endotoxin limit for the two examples would be

$$\text{EU} = \frac{5 \text{ EU/kg}}{25 \text{ mg/kg}} = 0.2 \text{ EU/mg}$$

$$\text{EU} = \frac{5 \text{ EU/kg}}{10 \text{ mg/kg}} = 0.5 \text{ EU/mg}$$

For devices, the endotoxin limit is 0.1 ng per milliliter of extract solution.

Four classes of drugs are exempted from the endotoxin limit defined by K/M:

1. Compendial drugs for which other endotoxin limits have been established
2. Drugs covered by new drug applications, antibiotic Form 5 and Form 6 applications, new animal drug applications, and biological product licenses for which different limits have been approved by the agency
3. Investigational drugs or biologics for which an IND or INAD exemption has been filed and approved
4. Drugs or biologics that cannot be tested by the LAL method example

Schmitz (62) reviewed all the progress of the establishment of endotoxin limits leading to the "Guideline on Validation of the *Limulus* Amebocyte Lysate Test as an End-Product Endotoxin Test for Human and Animal Parenteral Drugs, Biological Products, and Medical Devices" (35). This guideline contains a list of maximum doses per kilogram and the corre-

sponding endotoxin limits for a large number of aqueous injectable drugs and biologics on the market. The fifth supplement of USP XXII subsequently listed 185 monographs with new Bacterial Endotoxin Test requirements based on the maximum recommended total dose.

Sensitivity of LAL

LAL *sensitivity* is defined as the lowest concentration of a purified endotoxin that will produce a firm gel that will remain intact when inverted carefully after 1 hour of incubation at 37°C. (LAL sensitivity is also expressed as how many times its sensitivity is greater than the rabbit test.) In general, it seems to be well established that the LAL test is sensitive to picogram quantities of endotoxin, and that LAL is from 5 to 50 times more sensitive than the rabbit test to the presence of endotoxin, depending on the type of comparative study conducted.

Earlier studies by Cooper et al. (33) demonstrated that the LAL test was at least five times more sensitive to purified endotoxin than the rabbit test. This was later confirmed by Elin and Wolff (63). Improvements in LAL production and formulation methodology increased the sensitivity of LAL to be 10 to 50 times greater than the rabbit test (42,64). These numbers were based on a gel time of 1 hour and a rabbit test dose of 1 ml/kg.

Ronneberger (65) found that the LAL test gave similar results or was 10 times more sensitive than the rabbit test using lipopolysaccharides from different gram-negative bacteria (see Table 2.9). In more than 300 samples of drugs, plasma proteins, and other antigens, the LAL test and rabbit test gave similar results, although injection of a higher volume of test sample compensated for the lower sensitivity of the rabbit test.

Marcus and Nelson (31) have stated that the rabbit pyrogen assay will detect 1 to 10 ng of enterobacterial endotoxin, while the LAL test will detect 0.01 to 0.1 ng endotoxin per

Table 2.9 LAL Specificity and Sensitivity for the Detection of Lipopolysaccharides

LPS source	Minimum dose for positive rabbit response[a]	Minimum concentration for positive LAL reaction[b]
Salmonella typhi Type 58	1 ng	0.1 ng/0.1 ml
Salmonella abort. equi	10 ng	10 pg/0.1 ml
Lipid A of *Salmonella abort. equi*	100 pg	10 pg/0.1 ml
Salmonella minnesot.	10 pg	1 pg/0.1 ml
E. coli	10 ng	0.1 ng/0.1 ml
Klebsiella pneumoniae	1 ng	2 ng no reaction

[a] Three rabbits used.
[b] LAL source: Pyrogent (Mallinckrodt).
Source: From Ref. 65.

milliliter of solution. The ability of LAL to detect *E. coli* endotoxin in pyrogen-free distilled water was found to be 100 times more sensitive than the rabbit test (see Table 2.10) (66).

Lysate sensitivity will vary according to the commercial source of the lysate, as is the case with endotoxin sensitivity. Wachtel and Tsuji (67) tested six commercial lysate preparations against *E. coli* endotoxin. Sensitivity ranged from 0.03 to 0.003 ng/ml. Similar results were found with endotoxins extracted from *Salmonella typhosa*, *Serratia marcescens*, and *Shigella flexneri*. *Pseudomonas* sensitivity ranged from 10 ng/ml to as high as 500 ng/ml.

Twohy et al. (68) compared lysates from five LAL manufacturers (Associates of Cape Cod, Difco, Haemachem, M. A. Bioproducts, and Mallinckrodt). Using the gel clot method with nine different drug products and EC-5 endotoxin standard, these investigators found that end points varied among the LAL reagents from the different manufacturers. Lot-to-lot variability with LAL reagent from two manufacturers was

Table 2.10 Sensitivity of the Rabbit Pyrogen Test and of the *Limulus* Test in the Detection of *E. coli* Endotoxin

ng/ml	Rabbit pyrogen test[a]	Limulus test
500	Pyrogenic	Positive
50	Pyrogenic	Positive
5	Nonpyrogenic	Positive
0.5	Nonpyrogenic	Positive
0.05	—	Negative
0.005	—	Negative

[a] Dose: 1 ml endotoxin solution/kg body weight.
Source: From Ref. 66.

observed, as was the ability of some LAL reagents to change the pH of the drug product. These data support the fact that some LAL reagents are better suited for some drug products, than for other products, and that the LAL test must be revalidated for every drug product when LAL manufacturers and/or LAL lots are changed.

Sensitivity of LAL for endotoxin depends greatly on the vehicle in which the endotoxin is contained. For example, LAL can detect only 5 to 10 μg/ml endotoxin in plasma, whereas 0.05 μg/ml endotoxin was detectable in cerebrospinal fluid (69). The failure of LAL to detect known levels of endotoxin in human serum albumin and other protein solutions is well known (41). Many drug products inhibit the LAL test and severely retard its sensitivity. These inhibitions and limitations of the LAL test are discussed in the section, "Limitations."

Test Specificity

Whereas sensitivity is the ability of a test to give positive reactions in the presence of the material tested, *specificity* is the ability of a test to give positive reactions with only the material tested (31). The sensitivity of LAL toward endotoxin is

undisputed. However, its specificity in reacting solely with endotoxin is its most controversial characteristic.

In 1973, Elin and Wolff (63) first reported the possible lack of specificity of the LAL test for bacterial endotoxin. Substances found to cause lysate gelatin included thrombin, thromboplastin, ribonucleases, and polynucleotides such as polyriboadenylic acid and polyribouridylic acid. Wildfeuer et al. (69) found that peptidoglycans isolated from various gram-positive bacteria caused lysate gelatin. Positive reactions have been found between LAL and streptococcal exotoxins (70), synthetic dextrans (71), lipoteichoic acids (72), and the dithiols dithiothreitol and dithioerythritol (73). Intravenous immunoglobulin treatment has been found to produce false-positive LAL test results (74). Increasing the amounts of administered immunoglobulins increased the levels of LAL-reactive material in plasma.

Amibocyte lysate contains substances called (1–3) β-D-glucan–sensitive factors (75). These factors can activate LAL to produce false-positive results for the presence of endotoxin. Interestingly, very small amounts of β-glucan (1–1000 ng/ml plasma) will trigger gelation, while greater amounts of β-glucan (1 mg/ml plasma) will not (76).

Pearson and Weary (77) addressed these false-positive reactions caused by nonendotoxin substances. They concluded that such substances need not concern parenteral drug manufacturers because of one or more of the following reasons:

1. Many of the substances (including all of the synthetic substances) would not be found in a parenteral product.
2. The substance may be present, but not in sufficient concentrations to produce gelation in lysate or fever in rabbits.
3. The substance is a highly purified preparation that could not occur in production.
4. Results have not been confirmed by other researchers.
5. Because a negative LAL test result demonstrates the unequivocal absence of endotoxin, concern over false posi-

tives becomes a moot point with proper positive and negative controls.

Concerns over false negatives can be eliminated by the same validation process. For example, the clotting enzyme in LAL, coagulogen, is similar biochemically to trypsin. Trypsin, in turn, can initiate the gelation reaction (34). To be certain that a positive LAL test is due unequivocally to endotoxin contamination, adequate controls are used to demonstrate that substances like trypsin are not the cause of the gelation observed.

Advantages Compared to the USP Rabbit Test

Proponents of the LAL test claim that the test offers at least seven advantages over the use of the USP rabbit test for detecting pyrogens in parenteral injectable products and medical devices (25):

1. Greater sensitivity
2. Greater reliability
3. Better specificity
4. Less variation
5. Wider application
6. Use as a problem-solving tool
7. Less expense

The majority of these advantages are a direct result of the remarkable simplicity of the LAL test. Being an in vitro test requiring a minimal number of items to complete the test, LAL offers rapidity and reliability unmatched by an in vivo system.

Control of technique, handling, and external environmental factors is achieved much more easily with the LAL test. This, in turn, leads to minimized chances of error and variation in the testing results. The ease and adaptability of the LAL test allow it to be used in many different situations for which application of the rabbit test would be impractical or impossible. In fact, the need for a rapid, simple, and sensitive technique for pyrogen testing of extemporaneously prepared radiopharmaceutical preparations led to some of the earliest

applied research involving the LAL test in hospital pharmacy quality control. Other applications of the LAL test, rendered possible because of its unique advantages compared to the rabbit test, include pyrogen testing of in-process water for injection, bacterial and viral vaccines, antineoplastic agents, and drugs designed for intrathecal injection and validation of dry heat depyrogenation cycles. These applications are elaborated in the section. "LAL Test Applications."

The LAL test has become an acceptable substitute for the rabbit test in the in-process pyrogen control of plasma fractions (78). Four advantages given for substituting the LAL test in place of the rabbit test were as follows:

1. An in vitro test, when available, is employed rather than an animal test.
2. Results are available within 90 minutes after beginning the test procedure.
3. Tests that can be conducted with LAL when using the rabbit test would be senseless because of the time factor.
4. The LAL test is simple and inexpensive.

Fumarola and Jirillio (79) stated that, according to some 140 papers reported in the literature dealing with the LAL test as well as their own experience, the test is an acceptable, specific, rapid, and sensitive method for endotoxin assay of parenteral drugs and biological products and for in-process testing of parenteral solutions.

Researchers at Travenol Laboratories have published many articles providing data to support the superiority of the LAL test over the rabbit test for pyrogen testing of LVPs (25,30–32). Their arguments were summarized by Mascoli and Weary (25):

1. Pyrogens important in LVP products and devices are endotoxin in nature.
2. After tens of thousands of tests, an unexplained negative LAL test result–positive rabbit test result was never recorded.

3. Some endotoxin pyrogens detected by LAL were detected by rabbit tests.
4. In some cases, the rabbit test results only failed initially to detect pyrogens that were sometimes confirmed later by rabbit tests, but were always confirmed by initial LAL tests.

In a poll taken by M. J. Akers of quality control representatives from 10 pharmaceutical manufacturers of parenteral products and devices, 7 of 10 responded that they preferred the LAL test over the rabbit test. The advantages of the LAL test as reasons given for their preference were (in order of importance)

1. Greater sensitivity
2. Less variation
3. Quantitative results
4. Less time consuming
5. Less expensive
6. An easier test

The reader is directed to a nationwide survey of the biotechnology industry regarding practices also listed with endotoxin detection by the LAL assay (83).

Limitations of the LAL Test

Unquestionably, the LAL test fills the need for a simple, sensitive, accurate, and inexpensive method for detecting bacterial endotoxin. It certainly offers an excellent alternative or supplemental method of the official USP rabbit test for pyrogen. However, it is not without limitations or problems.

The greatest limitation of the LAL test is the problem of interference of the lysate-endotoxin interaction that is caused by a variety of drugs and other substances (84,87). Of the 10 quality control representatives from the parenteral industry we polled, 7 identified inhibition of the lysate-endotoxin interaction as the number one factor limiting the applicability of the LAL test. As discussed on pages 124–125, the LAL gela-

tion reaction is mediated by a clotting enzyme that is heat labile, pH sensitive, and chemically related to trypsin (89–90). Inhibition is caused by any material known to denature protein or to inhibit enzyme action. A representative listing of drugs and other substances known to modify or inhibit the lysate-endotoxin interaction is given in Table 2.11. Inhibition by many drug components can be overcome by dilution or pH adjustment. Of course, dilution reduces the concentration of the endotoxin and places greater demand on the sensitivity of the LAL reagent to detect diluted amounts of endotoxin.

Tests for inhibition or activation basically involve the use of positive controls. Product samples are "spiked" with known endotoxin levels, preferably the same levels used in standards prepared for sensitivity determinations. The end point of detection for the product sample should be no different from the end point for the standards series. In other words, if the lowest standard detectable level of endotoxin is 0.025 ng/ml, this level must also be detectable by the same lot of LAL reagent in the product sample. If inhibition is found to occur, serial dilutions of the product sample are made until the appropriate dilution is found that no longer modifies the gelation reaction.

Inhibition

According to Cooper (90), 30% of drug products do not inhibit the LAL test (producing an increase in the expected gelation onset time). Of the majority of products that do inhibit the test, 97% of the problems can be resolved because the inhibition is concentration dependent. Simple dilution usually can

LAL-endotoxin reaction.

LAL test inhibition is considered significant if the positive control varies by more than a twofold dilution from the standard in water. Inhibition acts on endotoxin, not the LAL reagent; that is, inhibition is often a failure to recover inadequately dispersed liposaccharide (aggregation of purified endotoxin).

Table 2.11 Examples of Small Volume Parenterals Reported to Markedly Inhibit the LAL Test

Inhibition overcome by more than one twofold dilution	Inhibition at maximum valid dilution
Aminophylline injection	Carbazochrome salicylate
Ascorbic acid and vitamin B complex injection	Cyclizine lactate
Chorionic gonadotropin	Diatrizoate meglumine and diatrizoate sodium
Clindamycin phosphate	Edetate disodium injection
Cyanocobalamin injection	Fluorescein sodium
Dicyclomine hydrochloride	Liver injection
Diphenydramine hydrochloride	Meperidine hydrochloride and promethazine hydrochloride
Dyphylline injection	Oxacillin sodium and other penicillin products
Ephedrine hydrochloride	Pentamidine isethionate
Fluorouracil	Peptonized iron large volume parenteral
Lidocaine hydrochloride	Sulfisoxazole
Lidocaine hydrochloride and epinephrine	Sulfobromophthalein sodium
Meperidine hydrochloride injection	Vancomycin hydrochloride
Mepivacaine hydrochloride and levonordefrin	Multi-vitamin injection
Promethazine hydrochloride	
Scopolamine hydrobromide	
Tetracaine hydrochloride	
Thiamine hydrochloride	

Source: C. W. Twohy, A. P. Duran, and T. E. Munson, *J. Parenter. Sci. Tech.*, *38*, 190–201, (1984).

Primary ways in which drug products inhibit the LAL test are by

1. Suboptimal pH
2. Aggregation or adsorption of control endotoxin spikes
3. Unsuitable cation concentrations
4. Enzyme or protein modification
5. Non-specific LAL activation

Other concerns or limitations of the LAL test are as follows:

1. LAL is dependable only for the detection of pyrogen originating from gram-negative bacteria.
2. Being an in vitro test, the LAL test cannot measure the fever-producing potential of endotoxin present in the sample.
3. The sensitivity of LAL varies appreciably with endotoxins from various microbial sources.
4. It is difficult to compare the sensitivity of the LAL test and the rabbit test because the rabbit assay is dose dependent, while the LAL test is concentration dependent.
5. Gel formation can be difficult to interpret and can be broken on the slightest vibration.
6. The LAL test is too sensitive in that it can detect endotoxin at levels below those required for producing fever in mammals. Yet, the FDA may enforce a level of sensitivity for the LAL test much greater than that for the rabbit test. In other words, a product that will consistently pass the USP pyrogen test may not pass the LAL test. Does this mean that the product is pyrogenic and harmful to humans?
7. Potential interferences from β-glucans.
8. Extensive studies are required to validate the LAL test as the final product pyrogen test.

Test Variability

There are several sources of variability that can affect the accuracy and reliability of the LAL test. It is for these reasons

that validation is so important and why the FDA produced its validation guideline for the LAL test (35). Pearson (15) and McCullough (91) have written excellent reviews on this problem.

1. Reagent Variability. There are significant differences in LAL reagent formulation from manufacturer to manufacturer (92). Although all LAL reagents are standardized to the USP RSE, both manufacturing processes and formulation differences account for variations seen in real-world endotoxin test situations. Major differences in reagent preparation include addition of the following: divalent cations, albumin, buffers, and surface-active agents. Some manufacturers allow the crude reagent to age, adjust coagulogen concentration, and perform chloroform extraction to remove inhibitors and increase sensitivity.

2. Method Variability. LAL reagents are designed specifically for optimal activity in each of the major LAL test systems. Thus, lysate–drug product compatibility may change when switching from one test method to another using the same lysate manufacturer.

3. Product Variability. It is well known that many parenteral products will interfere with the lysate-endotoxin reaction, although most of these interferences can be overcome by dilution (93).

4. Laboratory Variability. Type of glass and/or plasticware used (94), equipment calibration procedures, recalibration procedures, purity of water used, dilution procedures, and other different laboratory procedures all contribute to LAL test variability. As discussed previously, differences in handling (degree of agitation) and storage of parenteral products prior to LAL test analysis can markedly affect test results.

As a reiteration, to control all these sources of variability, the FDA wrote its guideline on validation of the LAL test (35). The guideline says, "The USP inhibition/enhancement tests must be repeated on one unit of the product if the lysate manufacturer is changed. When the lysate lot is changed, the two

lambda positive control is used to re-verify the validity of the LAL test for the product."

For an LAL reagent to be compatible with the FDA guidelines for LAL evaluation of drugs, devices, and biologicals and with the USP Bacterial Endotoxin Test, the reagent should have a stabilized sensitivity of 0.12 EU/ml. This sensitivity should be referenced to an *E. coli*–delivered LPS such as the USP RSE from *E. coli*. An LAL reagent should be buffered to accommodate small changes in pH of the test solution and be stabilized for divalent cations. The reagent also should be specific for endotoxin and should exhibit a clear and accurate end point.

LAL Test Applications

From a modest beginning of detecting endotoxin in blood, LAL test application has expanded into a variety of laboratory and clinical situations. New or improved usage of the LAL test appears in the literature on a monthly basis. Methodology has become more standardized, reference standards more accepted, and automatic instrumental analysis has been developed. LAL testing for endotoxin in the parenteral field has become standard practice.

At this time, the LAL test has been used as an indicator of endotoxin contamination in at least six different areas:

1. Pharmaceuticals
2. Biologics
3. Devices
4. Disease states
5. Food
6. Validation of dry heat cycles

The literature is massive with regard to LAL test applications in most of these areas. Not all published reports are discussed, but those with significant impact are described in the following sections. Reference 95 is a good source of articles dealing with applications of the LAL test.

Pharmaceuticals

The LAL test has overtaken the rabbit test as the main final pharmaceutical product release test for pyrogens. More than 200 USP monographs now contain endotoxin limits using the LAL test.

Radiopharmaceuticals represent a special class of parenteral medications for which the LAL test offers unique advantages in the detection of pyrogen contamination. Many radiopharmaceuticals are prepared extemporaneously, such as technetium 99m (^{99m}Tc), which has a biological half-life of only 6 to 7 hours. The LAL test, because of its short time for testing, low volume requirements, and low cost, obviously is the preferred method for pyrogen detection in radiopharmaceuticals. DeMurphy and Aneiros (96) used a micro-LAL test method for pyrogen detection in 204 radiopharmaceuticals, including pertechnetate, sulfur colloid, pyrophosphates, pyridoxylidenglutamate, human serum albumin, and human albumin macroaggregates. They concluded that the test proved to be economical, easy, rapid, sensitive, and reliable. The test was incorporated into the routine quality control program not only for radiopharmaceuticals, but also for all parenteral fluids and solutions used in kit preparation within their nuclear medicine department.

Rhodes and Croft (97) listed six reasons why the LAL test is preferred over the rabbit test for pyrogen testing of radiopharmaceuticals and reagent kits:

1. It is more sensitive.
2. It is faster.
3. It requires smaller amounts of test material.
4. Both positive and negative controls can be performed along with each test.
5. It does not generate radioactivity in the rabbits so it is preferred from a radiologic safety point of view.
6. It is less expensive and easier to store.

Antineoplastic agents are another class of parenteral medication for which the LAL test provides marked advan-

tages over the USP rabbit test. Endotoxin is an expected contaminant of the enzyme L-asparaginase (34) because it is obtained from cultures of *E. coli* ATCC 9637. However, the USP pyrogen test cannot be used to detect endotoxin in this preparation because the rabbit is one of the species extremely susceptible to the toxic effect of the enzyme (98). L-Asparaginase and bleomycin contain as much as 50 ng/ml endotoxin (99). It is suspected that this contaminant is the cause of the adverse effects seen in patients following administration of these agents. The LAL test sensitivity characteristics aid in evaluating the techniques applied to reduce or eliminate the endotoxin level in these agents.

The LAL test has been used to detect the presence of bacterial endotoxin in 12 chemotherapeutic agents (100). Relative concentrations of endotoxin ranging from 0.1 to 63 ng/ml were detected in individual lots of the following drugs: L-asparaginase, 5-azacytidine, bleomycin, DTIC, antinomycin D, adriamycin, and vinblastine. On the other hand, all lots of the following antineoplastic agents contained = 0.1 ng/ml endotoxin: cytosine arabinoside, cyclophosphamide, daunorubicin, vincristine, and streptozotocin. The authors concluded that the LAL test is a rapid and specific method for detection of small amounts of bacterial endotoxin contaminating parenteral preparations of antihumor agents.

Antibiotics are known to inhibit the LAL test at the product concentrations used in human or animal dosages. In most cases, however, adequate dilution of most of these products above the minimum valid concentration (MVC) will provide noninhibitory conditions for successful application of the LAL test. Case et al. (101) tested 28 antibiotics with the LAL assay to determine their noninhibitory concentrations (NICs). Most of the antibiotics tested could be diluted to NICs above the MVCs. Five antibiotic products presented problems. Cefamandole nafate and neomycin sulfate had NICs very close to their MVCs (1.6:0.8 mg/ml and 0.2:0.16 mg/ml, respectively). Polymyxin B and colistimethate contained too much endotoxin to permit determination of their NICs. The NIC of tetracycline

hydrochloride was dependent on the initial concentration of the antibiotic. If the initial concentration of tetracycline was 5 mg/ml, dilution to 0.16 mg/ml produced a noninhibitory concentration that was less than the MVC for tetracycline. However, a concentration of 0.5 mg/ml, when diluted, produced an NIC that was about the same as the MVC. The reason for this difference probably was the amount of NaOH required to adjust the pH of this very acidic antibiotic solution (pH 2.8). The greater amount of base required to increase the pH of the 5.0 mg/ml product probably caused too high a sodium ion concentration for the LAL test to overcome.

Other pharmaceutical preparations for which the LAL test has proven itself as a final product release test for pyrogens include LVPs (102), intravenous fat emulsions (103), iron dextran (104), and most of the drug products listed in Table 2.11. Despite the need for dilution to eliminate the inhibitory effects of many small-volume parenteral drug products, the LAL test is at least equal to or more sensitive than the USP pyrogen test.

Pharmaceuticals administered by the intrathecal route represent a drug class most urgently in need of the LAL test for endotoxin detection (42). Such pharmaceuticals include (a) dyes such as methylene blue and fluorescein for detecting cerebrospinal fluid (CSF) leakage, (b) contrast media for visualization of CSF pathways, (c) cancer chemotherapeutic agents such as methotrexate for treatment of leukemic meningitis, (d) antibiotics such as gentamicin for septic meningitis, and (e) radiopharmaceuticals for radionuclide cisternography, a procedure by which a small volume of radiotracer is administered intrathecally to study CSF dynamics by means of nuclear imaging devices. Endotoxin has been shown to be more toxic following intrathecal injection compared to intravenous injection. For example, Bennett and coworkers (105) demonstrated in animals that instillation of endotoxin into intrathecal spaces was at least 1000 times more potent in producing a febrile response than the intravenous route.

The USP rabbit pyrogen test for intrathecal drugs has

been shown to be insufficiently sensitive to serve as a screening test for endotoxin contamination of these drugs (42). Thus, the LAL test should replace or at least supplement the USP pyrogen test for drugs intended to be administered into CSF.

Biologics

The FDA's Bureau of Biologics (BoB) (now Center for Biologics Evaluation and Research) in 1977 published conditions under which the LAL test can be applied as the end-product pyrogen test for biologics (see page 124). The main requirement involves validating that the LAL test and rabbit test are at least equivalent for each product undergoing pyrogen testing. Comparison to the rabbit test is no longer required provided that the test is validated to USP and FDA guidelines.

The LAL test has been used both for end-product testing and for solving problems during the manufacturing of blood products and plasma fractions. Expediency, sensitivity, and quantitation of endotoxin levels are three advantages of using the LAL test rather than the rabbit test. A comparison of the two pyrogen tests as they are applied to various biological substances was reported by Ronneberger (65), and an example of Ronneberger's data is given in Table 2.12.

LAL assays have been demonstrated as satisfactory for three primary biological substances: human serum albumin (77,106), plasma fractions (65,78,107), and vaccines (108). However, human serum in toto inhibits the LAL gelation reaction with spiked endotoxin unless modifications in the test procedure are incorporated. Human serum contains a single protein (designated LPS-1) that inactivates LPS and inhibits the gelation reaction (109). Other inhibitors are present in serum, such as two α-globulins (110) and a serum globulin esterase (111). Another variable is that the levels of substances inhibiting endotoxin probably vary not only from person to person, but also in a patient during various stages of illness associated with gram-negative infections (112).

Three methods have been reported that are capable of removing these serum inhibitors. They include extracting the

Table 2.12 Comparison of LAL and Rabbit Tests on Various Materials

Material	No. of samples	Dose rabbit ml/kg LV.	Rabbit[a] +	Rabbit[a] −	LAL[b] dose 0.1 ml +	LAL[b] dose 0.1 ml −	Rabbit − LAL +
Hemaccel plasma substitute	35	10	2	33	2	33	0
Physiologic saline	21	10	2	19	2	19	0
Aqua dest.	25	10	3	22	3	22	0
Albumin, human	65	3	21	44	31	34	10
Gammaglobulin	31	1	8	23	16	15	8
$F(ab)^2$-fragment	27	3	8	19	12	15	4
Plasma protein solution	18	3	44	14	9	9	5
Fibrinogen	6	1.5	1	5	3	3	2
Factor XII	10	1	2	8	2	8	0
Proteinase inhibitor Antagosan	9	2	1	8	1	8	9
Interferon	6	1	4	2	5	1	1
Streptokinase	35	1	3	32	5	30	2
Neuraminidase	12	1	4	8	6	6	2
Vaccines div.	45	0.5–10	24	21	26	21	2
Total	345	0.5–10	127	258	123	224	45
Plasma human treated chloroform	28	1	18	10	5	23	

Source: Ref. 65.

[a] Rabbit + 2 LAL − = Plasma 13.

[b] LAL: Pyrogent. Other samples: 4.

serum with chloroform (113), adjusting the plasma pH (114), and combining the application of heat and serum dilution to overcome the inhibiting capacity of the inhibitors (115).

Baek (116) reported an immunoelectrophoretic assay that greatly improves the sensitivity of LAL for LPS detection in various biological fluids, including plasma. The assay method is based on the preparation of a monospecific antibody against coagulogen, the clottable protein formed in the LAL-endotoxin reaction (see the section, "Reaction Mechanism"). As coagulogen splits into coagulin and C-peptide, the antigenicity of the cleaved coagulogen is lost, and this is expressed by a diminished migration of the protein on the rocket immunoelectrophoresis (RIE) plate. The method increased LPS detectability in plasma by 1000 times, that is, from 1 ng LPS/ml of plasma by visual LAL testing to 1 pg LPS/ml plasma using RIE.

Devices

In a *Federal Register* notice on November 4, 1977 (42FR, 57749), the LAL test was approved by the Bureau of Medical Devices of the FDA (now the National Center for Devices and Radiological Health, CDRH) as a suitable test to replace the USP pyrogen test for final release of medical devices. As with the biologics, the manufacturer may use the LAL test as a final release test for devices only after meeting four conditions:

1. Demonstrate the equivalence of the LAL test and rabbit test for each device.
2. Document the proficiency in applying the LAL test.
3. Describe LAL test methodology in detail.
4. Determine acceptance limits for the applicable device products.

All validation data required prior to releasing devices labeled nonpyrogenic based on the LAL test must be kept on file at the manufacturing site and be available for FDA inspection (117). If a manufacturer plans to use LAL test procedures that differ significantly from FDA guidelines, then the manufac-

turer must submit either a 510(k) or a premarket approval supplement.

Hundreds of device manufacturers in the United States as well as in foreign countries have taken advantage of the opportunity to replace the rabbit test with the LAL test. The major barrier that must be solved for a device manufacturer to receive regulatory approval to use the LAL test involves the validation of the equivalency of the two pyrogen test methods.

Representatives of HIMA took the initiative to propose guidelines (118) and conduct studies (119) to help the medical device manufacturer successfully design and carry out procedures for LAL pyrogen testing. The guidelines suggested are summarized in Fig. 2.15. The collaborative study conducted under HIMA auspices evaluated the pyrogenicity of *E. coli* 055:B5 from Difco Laboratories (Detroit, MI). Using 12 rabbit

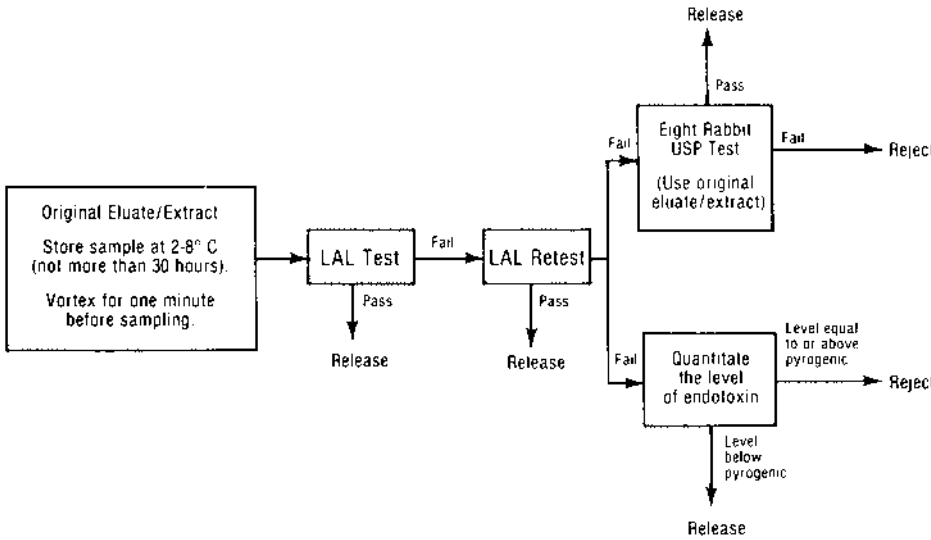

Fig. 2.15 Schematic flow chart of the guidelines proposed by HIMA for LAL pyrogen testing of medical devices. (From M. Weary and F. Pearson, Pyrogen testing with *Limulus* amebocyte lysate, *Med. Devise Diag*., Nov. 1980, Cannon Communications, Inc.).

colonies provided by device manufacturers, contract testing laboratories, and the FDA, the average pyrogenic dose of *E. coli* 055:B5 endotoxin was found to be 0.157 ng/ml (1.57 ng/kg for 10 ml/kg injected dose). The lower 95% confidence level was 0.1 ng/ml, meaning that if 8 rabbits were administered this concentration of endotoxin, 4 rabbits would fail the pyrogen test at this dose. Therefore, any device manufacturer wishing to use the LAL test must validate the ability of their test procedure to detect Difco *E. coli* 055:B5 endotoxin at levels at least equivalent to 0.1 ng/ml (1.0 ng/kg dose).

CDRH requires the following sampling guidelines to be used for different device lot sites:

Two devices for lots less than 30
Three devices for lots of 30–100
Of lots above 100, use 3%, up to a maximum of 10 devices per lot

The recommended volume of nonpyrogenic rinsing fluid per device is 40 ml. If 10 devices are rinsed, then the total rinsing extract pooled is 400 ml. If a rinse volume greater than 40 ml per device is required, a more sensitive LAL end point should be used.

Disease States

Because endotoxins are associated with gram-negative bacteria, diseases caused by these bacteria conceivably can be diagnosed by the LAL test. A partial list of gram-negative bacteria is given in Table 2.13 along with diseases associated with these organisms.

Endotoxemia is a low-grade infection of the intestinal tract caused by bacterial endotoxins. Endotoxemia can result in endotoxic shock, which is a common cause of morbidity and mortality in hospital patients. Detection of endotoxemia by the LAL test was first assessed by Fossard et al. in 1974 (120). They concluded that the LAL test is a simple, rapid, and reliable method for detecting endotoxemia. Early detection permits early and vigorous treatment of the infection, with the LAL test used to monitor the effectiveness of the treatment.

Table 2.13 Gram-Negative Bacteria and Diseases Associated with These Microorganisms

Cell shape	Genus	Disease
Cocci	*Neisseriae*	Gonorrhea
		Meningitis
Rods	*Pseudomonas*	Wound, burn infection
		Pneumonia
		Eye Infection
	Escherichia	Gastroenteritis
		Urinary tract infection
	Shigella	Dysentery
	Proteus	Urinary tract infection
	Hemophilus	Infantile meningitis
		Chronic bronchitis
	Salmonella	Typhoid fever
		Food poisoning
	Brucella	Animal infections

LAL assay found high plasma endotoxin levels in patients suffering from sepsis, malignant tumors, leukemia, and decompensated liver cirrhosis (121). A modification of the LAL test was required to eliminate interference factors located in platelet-rich plasma or serum. A simple addition of perchloric acid to plasma in a final concentration of 1.25% eliminated the inhibitors. The LAL assay currently is being applied in studies trying to determine the correlation of endotoxin levels and various diseases.

Although not sanctioned by the FDA, the LAL test has proven to be very useful in the diagnosis of meningitis caused by pyrogenic radionuclide substances used in cisternography (122) and meningitis resulting from gram-negative bacteria (123–127). The sensitivity, reliability, and rapidity of the LAL method are vitally important because of the serious toxicity problems associated with pyrogens and bacteria in CSF (128). The LAL test of CSF was found to be clinically useful in neonates suffering from gram-negative infection (129).

The LAL test has been used successfully and holds important future applications in diagnosing and monitoring such various disease states as gingival inflammation (130), bacteriuria (131,132), postanesthesia hepatitis (133), urinary tract infections (134), mastitic milk (135), gram-negative sepsis resulting from burns (136) and other causes (137), gonococcal cervicitis (138), peritonitis (139), and gonorrhea (140,141).

Food

LAL testing has reached into some areas of food and drinking water processing. LAL has been used to determine endotoxin levels in drinking water (142,143), marine environment (144), sugar (145), and ground beef (146). The levels of endotoxin provide evidence of the microbial quality of the food material. For example, LAL found 10 ng/g endotoxin for both white and beet raw sugar, while 100 ng/g endotoxin were found in imported cane raw sugar (145).

Other Applications

The LAL test has proven to be a valuable test for the detection of endotoxin extracted from surgeons' sterile latex gloves (147) and operating nebulizers used in respiratory therapy (148).

Validation of dry heat sterilization and depyrogenation cycles based on the destruction of endotoxin can be accomplished through the employment of the LAL test (149,150). This could not be accomplished practically using the USP rabbit test. This has resulted in an FDA requirement for a three-log reduction in endotoxin levels in materials being dry heat sterilized (151).

Analogous to biological indicators used for validation and routine monitoring of sterilization processes, there are now "endotoxin indicators" that can be used in the validation and routine control of endotoxin reduction processes (152).

MODIFICATIONS OF RABBIT AND LAL TESTS FOR PYROGENS

Ultrafiltration, using a membrane having a fraction molecular weight of 10,000, has been used to separate endotoxin contam-

ination from injectable solutions of sodium ampicillin (153). The filtrate solution contained endotoxin, while the antibiotic remained entrapped on the filter. Samples of the filtrate were tested for pyrogenicity by the rabbit pyrogen test, the LAL gelation test, and the chromogenic assay method. All three tests were positive for endotoxin. Without ultrafiltration, the presence of sodium ampicillin interferes with rabbit, LAL, and chromogenic detection of endotoxin (154). Ultrafiltration also allows concentration of endotoxin in drug preparations, facilitating detecting of minute (picogram) amounts of endotoxin.

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3

Particulate Matter Testing

INTRODUCTION

No quality control test, parenteral or nonparenteral, presents more difficulties for quality control specialists than inspection and analysis of injectable solutions for the presence of foreign particulate matter. The oldest, yet most commonly used, test for particulate matter evaluation involves human visual examination. Such examination is subjective, time consuming, and limited in the types of parenteral products and containers that can be inspected. This has stimulated many studies regarding ways of not only improving efficiency of human inspection, but also developing and improving methods of detecting particulate matter electronically.

The U.S. Pharmacopeia (USP) requirement for injectable products specifies that "each final container of injection be subjected individually to a physical inspection, whenever the nature of the container permits, and that every container whose contents show evidence of contamination with visible foreign material be rejected" (1). Additional specifications are required for subvisible particulate matter content and analy-

sis in large-volume injections (LVIs) for single-dose infusion and in small-volume parenterals (2).

The European Pharmacopeia (EP) contains sections on subvisible particulate contamination and visible particulate contamination (3).

Why are injectable solution products to be free of visible evidence of particulate matter? Primarily, lack of particulate matter conveys a clean, quality product, indicative of the high-quality standards employed by the product manufacturer. Moreover, in recent years, particulate matter has become known as a potential hazard to the safety of the patient undergoing parenteral therapy. While there still seems to be a lack of sufficient clinical data to incriminate particles as producers of significant clinical complications during parenteral therapy, it is a universal belief in the health care field that particulate matter does present a clinical hazard and must be absent from the injectable solution.

The aim of this chapter is to concentrate on particle testing methods in the quality control analysis of parenteral solutions. Two primary methods of particulate analysis—visual inspection and electronic particle counting—are discussed in detail. Barber's recent edition (4) represents an encyclopedic reference for particulate matter testing.

BACKGROUND OF PARTICULATE MATTER CONCERNS IN PARENTERAL PRODUCTS

It is interesting to realize that all the attention given today to the problems and analysis of particulate matter in parenteral products did not exist before the 1940s. After the inclusion of the first injectable product in the USP (12th edition) in 1942, Godding (5) was the first individual to publish an article concerning the need for standards in the visual inspection of particulate matter. The 13th edition of the USP (6) gave a detailed method for inspecting an injectable solution against a white-and-black background using a light intensity between 100 and 350 footcandles at a distance of 10 inches. Interestingly, the

method described in the 13th edition is still widely used in manual inspections for evidence of visible particulate matter.

The "rule-of-thumb" standard that a person with 20/20 vision under inspection conditions should be able to detect approximately 30-μm particles came from a report by Brewer and Dunning (7). This detection limit has persevered since 1957, although later research suggested that inspectors should actually be able to see particles in the size range of 20 μm (8).

In the early 1950s, a number of reports began citing evidence of biological hazards produced by foreign injected materials. Among the materials found to cause pulmonary granulomata or emboli were cotton fibers (9) and cellulose (10). Glass particles and their potential hazard was studied by Brewer and Dunning (7) and later by Gnadinger (11), but no evidence of foreign body reactions in animals were found. These and other reports led to the classic works done by Garvan and Gunner published in 1963 and 1964 (12,13). These Australian physicians showed that foreign body granulomas could be produced experimentally in the lungs of rabbits following the administration of 500 ml saline solution contaminated with visible particulate matter. Most commercial intravenous solutions inspected contained particle contamination and the source of most of the particles was attributed to the rubber closure. For every 500 ml of particle-contaminated intravenous solution injected into a rabbit, 5000 granulomas appeared in the lungs. Garvan and Gunner further found that similar granulomas appeared in the postmortem examinations of the lungs of patients receiving large volumes of intravenous fluids. Their comments included the possibility that postoperative pulmonary infarction was a result of particulate thrombosis. The repercussions of Garvan and Gunner's reports have stimulated numerous studies that continue to this day on the analysis and potential clinical hazards of particulate matter.

A collaborative study conducted by the Pharmaceutical Manufacturers Association (14) involved the intravenous in-

jection of varying quantities and sizes of inert polystyrene spheres into hundreds of rats, then performing necropsies at various periods of time from 1 hour to 28 days following injection. The results were as follows:

1. Of 18 rats injected with 8×10^6 particles per kilogram at a particle size of 40 μm, 13 died within 5 minutes.
2. Rats showed normal blood studies, organ weights, and pathologic criteria after being injected with either 8×10^6 particles at a size of 0.4 to 10 μm or 4×10^5 particles per kilogram of 40 μm particle.
3. Particles in the 4-μm size range were found in the lung, liver, and spleen.
4. Particles in the 10-μm size range were found in the lung primarily, although particles were found in five other organs.
5. Particles in the 40-μm size range were found in the lungs and myocardial tissue.

It was concluded that nonreactive particles administered intravenously over a broad size range and up to dosages that produced death were without clinical or tissue toxicity. Much disagreement resulted over this conclusion, especially because of the artificial nature of the type of particle studied. However, the same size-dependent localization of particles in different organs was found in the case of glass particles derived from breaking the necks of glass ampules (15). Large particles (>20 μm) were retained mostly in the lungs of mice, while smaller particles (5–10 μm) were found in the liver, spleen, and kidney. No glass particles were found in the brain.

The potential hazard of particulate matter has been implied in a number of reports, two of which are cited here. In a study of 173 patients undergoing cardiac catherization and/or surgery, 14 (8%) had fiber emboli in routine autopsy sections (16). The embolized fiber often resulted in narrowing or occlusion of the involved blood vessel. Three cases of myocardial infarction were associated with embolic fibers. Fibers were believed to have originated from various materials used

in surgery and from drug solutions. It was concluded that particulate matter is a hazard, and all steps must be taken to prevent its inadvertent administration.

A second critical report implicating the hazards of particulate matter was the work published by DeLuca et al. (17). In a repeated double-blind study of 146 patients, a significant reduction in the incidence of infusion phlebitis was seen when patients were administered intravenous fluids filtered through an in-line 0.45-μm filter. Other studies, as reviewed by Turco and King (18), have supported this finding.

Only ancillary reports have appeared in the literature in the past few years regarding new findings on the clinical hazards of particulate matter. These include a report by Stein and Vu (19) that a piece of rubber, apparently cored out of the rubber closure of a vial, caused some interference during cataract surgery.

Barber (20) cites the review by Pesko (21) as the most recent and best focused review of the literature on the hazards of particulate matter. Some interesting facts from this article include the following:

Size, number, rate of introduction, and type of particle entering the bloodstream will all contribute to what harm, if any, the particle(s) actually produce. Some particles might cause allergic reactions.

The health condition of the person receiving solutions containing particulate matter also greatly matters with respect to potential harm of these particles.

Some particles will cause an inflammatory response. The potential harm of this inflammatory response depends on where the particles end up in the body and, if they are located in a vital organ, what is that organ's capacity to compensate for the insult caused by the foreign matter.

Freedom from visible evidence of particulate matter is a basic, essential characteristic of injectable products. Such a characteristic imparts three significant qualities to the product:

1. Significance to the manufacturer—lack of particulate matter indicates good production technique and a high-quality product.
2. Significance to the user—lack of particulate matter indicates a clean product that is safe to the patient and conveys high-quality standards employed by the manufacturer of the product.
3. Clinical significance—lack of particulate matter indicates the lack of potential hazards resulting from particles entering the circulatory system, although controversy still exists regarding the hazards of particulate matter (23).

NATURE AND SOURCE OF PARTICULATES

Anything that directly or indirectly comes in contact with a parenteral solution, including the solvent and solutes composing the solution itself, represents a potential source of particulate contamination. Table 3.1 lists common sources of particulates found in parenteral solutions.

The type and approximate size range of particulates found in parenteral products are listed in Table 3.2. The smallest capillary blood vessels are considered to have a diameter of approximately 7 μm. Thus, all particles having a size equal to or greater than 7 μm can conceivably become entrapped in and occlude a blood capillary. Most particulates, as seen in Table 3.2, potentially can be this size and, obviously, represent a hazard to the health of a patient administered parenteral medications containing these contaminants.

It seems that, regardless of whatever painstaking procedures are undertaken to eliminate particle contamination, parenteral solutions always contain a certain degree of particulate matter. It is always an uncertainty whether the particles originated during the manufacturing and packaging process or were introduced during the analysis of the solution for the presence of particulates. The emphasis on technique in the analysis of particulate matter has been stressed by Draftz and Graf (25), McCrone (26), and Barber (27). It is imperative that

Table 3.1 Common Sources of Particulate Matter

1. Chemicals
a. Undissolved substances
b. Trace contaminants
2. Solvent impurities
3. Packaging components
a. Glass
b. Plastic
c. Rubber
d. Intravenous
4. Environmental contaminants
a. Air
b. Surfaces
c. Insect parts
5. Processing equipment
a. Glass
b. Stainless steel
c. Rubber
d. Rust
6. Filter fibers
7. People
a. Skin
b. Hair
c. Gowning

particles seen in solutions have not originated during the particle measurement and identification procedures.

THE REALITY OF PARTICULATE MATTER CONTAMINATION IN PARENTERAL SOLUTIONS

While it is desirable to prepare and use parenteral products completely free from particulate matter, it must be admitted that this ideal state is not possible. All parenteral products contain some level of particulate matter contamination. The question is, "How many particles, of what type, and what size?"

Table 3.2 Type and Approximate Size Range of Extraneous Materials Reported in Parenteral Solutions

Material	Approximate size range (μm)
Glass	1
Metal	1
Rubber	1 to 500
Starch	1
Zinc oxide	1
Whiting	1
Carbon black	1
Clay	1
Diatoms	1 to 5
Bacteria	2
Fungi and fungal spores	20
Insect parts	20
Cellulose fibers	1 to 100
Trichomes	10
Miscellaneous crystalline material	1
Talc	1
Asbestos fibers	1 to 100
Unidentified fibers	1

Source: Ref. 24.

Barber (4) addresses this question throughout his book. Below are excerpts taken from his book.

> Particulate matter present in parenteral solutions and medical devices has been an issue in the pharmaceutical industry since the introduction of injectable preparations and remains unavoidable, even with today's well controlled manufacturing processes. (p. 2)
>
> Correctly or incorrectly, the particulate matter burden of a product has been taken by some healthcare practitioners, academic investigators, and regulatory personnel as an indicator of overall product quality. This is

unfortunate, since particulate matter is, realistically, only a single parameter by which product suitability or conformity may be judged. (p. 2)

The practice content of IV fluids in plastic containers has been repeatedly found to be lower than that of glass containers. (p. 14)

There are three very important concepts embodied in the definitions provided by the USP, JP, and BP:

1. Particulate matter currently exists at extremely low levels in injectable products so that there is no demonstrable evidence of adverse patient effects.
2. The material cannot be monotypic, but rather results from a variety of sources inherent in a GMP-controlled production process.
3. The material is not amendable to chemical analysis due to the small mass that it represents and its heterogeneous composition. Thus, the appropriate analytical enumeration of this material must be sensitive physical tests that detect size and quantitate the material based on its optical properties. (pp. 20–21)

The occurence of low numbers of heterogeneously sourced particles is inevitable in the manufacture of injectable products and medical devices. (p. 22)

The USP requires that injectables be "essentially free" of visible particulate matter. The allowable particle burden of units tested must ultimately be judged with respect to the acceptable small quantity of visible particulate matter that may be present in units produced by parenteral manufacture under cGMP conditions. The most important aspect of the visual inspection procedure is the detection of any particulate matter that is related to solution degradation or any particulate matter present in sufficient quantity or of sufficient size to constitute a non-GMP condition. (pp. 261–262)

Despite rigorous cleaning procedures either at the vendor's plant or in-house, glass vials and stoppers occasionally will bear or contain a single visible particle following filling, stoppering or lyophilization, and these par-

> ticles may become free in the solution. Such single visible particles of random isolated occurrence are analogous to the "allowable" particle burden under USP <788>. A unit bearing such an isolated single visible particle must be rejected. On the basis of the low level of occurrence of such visible particulates in the batch of manufactured materials from which the unit came, however, the batch may be still be considered essentially, substantially, and practically "free" of visible particulate material. (p. 262)
>
> It is important for the pharmaceutical industry and regulators to recognize that there are no particle-free parenteral solutions. There is, further, no evidence of any patient issue related to infusion of a small number of inert particles with the current or previous USP limits. (p. 273)

PARTICULATE MATTER STANDARDS

The first reference to particulate matter in the USP occurred in the eighth edition in 1905 (28,29). Diphtheria Antitoxin, a hypodermic injection product, was described as a "transparent or slightly turbid liquid." Not until 1936, in the National Formulary (NF), sixth edition, was the term "clearness" defined for parenteral products (30): "Aqueous Ampul Solutions are to be clear; that is, when observed over a bright light, they shall be substantially free from precipitate, cloudiness or turbidity, specks of fibers, or cotton hairs, or any undissolved material" (24).

The words *substantially free* caused interpretive difficulties; thus, in 1942, the NF, seventh edition, provided a definition: "Substantially free shall be construed to mean a preparation which is free from foreign bodies that would be readily discernible by the unaided eye when viewed through a light reflected from a 100-watt mazda lamp using as a medium a ground glass and a background of black and white." It was also in 1942 that the 12th edition of the USP contained its first particulate matter standard:

> Appearance of Solution or Suspension Injections which are solutions of soluble medicaments must be clear, and

> free (note the absence "substantially") of any turbidity or undissolved material which can be detected readily without magnification when the solution is examined against black and white backgrounds with a bright light reflected from a 100-watt mazda lamp or its equivalent.

The requirement that every injectable product in its final container be subjected individually to visual inspection appeared in the 13th edition of the USP (5). This requirement has remained essentially unchanged; as the 25th edition states: "Good pharmaceutical practice requires also that each final container of injection be subjected individually to a physical inspection, whenever the nature of the container permits, and that every container whose contents show evidence of contamination with visible foreign material be rejected" (1).

The problem with the above USP statement lies with the word *visible*, which has the connotation of particles being seen with the unaided eye. The unaided eye can discern, at best, particles at sizes of about 40–50 μm. Detection of smaller particles cannot be accomplished assuredly with the USP physical inspection test. Health care professionals became increasingly concerned about the aspect of intravenous solutions, especially large-volume parenterals, contaminated with particles too small to be seen with the unaided eye, yet still hazardous when introduced into the veins of a recumbent patient. In the mid-1970s, the USP and Food and Drug Administration (FDA) cosponsored the establishment of the National Coordinating Committee on Large-Volume Parenterals (NCCLVP). The NCCLVP then established a subcommittee on methods of testing for particulate matter in LVIs. Ultimately, the efforts of this subcommittee resulted in the establishment of the USP microscopic assay procedure for the determination of particulate matter in LVIs for single-dose infusion and set upper-limit acceptable particle standards at particle sizes of 10 μm and 25 μm (31). These two sizes were also subsequently used as size standards for particulate matter in small-volume injections.

The USP standards for LVIs came after standards were first established in Australia and Britain. The Australian standards were based on research by Vessey and Kendall (32); their results are reported in Table 3.3 along with the upper-limit particle specifications in the British Pharmacopoeia (BP) and USP.

Because of the widespread acceptance of instrumental methods for counting and sizing particles, several alternatives to the present LVI particle limit specifications seen in Table 3.3 have been proposed. The National Biological Standards Laboratory (NBSL) of Australia adopted an approach that depends on the mean and standard deviation of the results from 10 individual containers. This approach takes into account the usually wide variation in particle counts measured from container to container. As seen in Table 3.3, under the Australian standards, no more than 100 and 2 particles per milliliter at particle sizes of 5 μm and 20 μm, respectively, are permitted

Table 3.3 Particulate Matter Standards in Various Compendia Compared to Those Suggested by Vessey and Kendall

Particle size (≥μm)	Vessey and Kendall (32)	Australia[a] (33)	British Pharmacopoeia[b] (34)	U.S. Pharmacopeia[c] (2)
2	1000	—	1000 (500)	—
3.5	250	—	—	—
5	100	100	100 (80)	—
10	25	—	—	50
20	—	2	—	—
25	—	—	—	5

[a] Mean count of at least 10 containers using light-blockage method. See text for additional specifications.

[b] Particle standards apply only to specific solutions using conductivity (Coulter Counter). Numbers in parentheses refer to particle limits if light blockage (HIAC) is used.

[c] Particle standards apply only to large-volume parenterals. Particle numbers and size determined by microscopic methods unless electronic methods have been shown to have equivalent reliability.

in LVI solutions. However, the Australian standards also state that, at the 5-μm particle size level, the sum of the mean and twice the standard deviation is not more than 200; that is,

$$\bar{x} + 2s \leq 200$$

and at the 20 mm size, the sum of the mean particle count and twice the standard deviation is not more than 4:

$$\bar{x} + 2s \leq 4$$

The Australian approach combines the mean values of 10 containers. Hailey et al. (35) suggested the use of a statistical limit that would account for the mean and standard deviation of particle counts obtained for *each* of the 10 containers used. Their proposal uses a term called statistic S_T, which is defined mathematically as

$$S_T = [\sum(\bar{x} - T)^2/n]^{1/2}$$

where x is the mean value of n results, and T is the target value (the desired value of the system being measured). For LVIs, the desired value would be zero (no particles), so the above equation would become

$$S_T = [\sum\bar{x}^2/n]^{1/2}$$

$$S_T = [S^2(n - 1)/n + \bar{x}^2]^{1/2}$$

where S is the standard deviation. The advantage of the S_T approach over the Australian draft standard is the increased stringency of the S_T requirement on samples near the limit for mean particle count and having large standard deviation. For example, if $\bar{x}$ for 10 containers were 100.0, $\bar{x} + 2s$ were 134.4, and S_T were 101.3, the sample would pass the Australian Test $\bar{x} + 2s$ than 200), but fail the S_T test (value $\geq$ 100). The disadvantage of the S_T test is that it is based on a target of zero, a level of cleanliness that can never be achieved. However, to use any other target value would introduce additional problems, as discussed by Hailey et al. (35). The authors conclude by stating that an S_T target of zero does not add unrealistic

constraints on manufacturers, but it does reduce the risk of passing a lot of LVI solution having a few containers that are extremely contaminated with particles.

Groves and Wana (36,37) proposed that a single numerical value, the index of contamination C, be adopted as a standard for accepting or rejecting LVI fluids. C is obtained from log particle size–log particle number plots of solutions measured by Coulter Counter and light blockage methods in which the results cross over a size threshold of around 6.0 μm. Using the log-log relationship for the BP limits between 2 μm and 5 μm and extending the time to 6.0 μm, the extrapolated number of particles equals 63.244. From previous derivations outlined by Groves and Wana (37), the index of contamination may then be defined as

$$C = (\ln N_{1.0} - \ln 63.244)/m$$

where $N_{1.0}$ is the estimated number of particles per unit volume at a size threshold of 1.0 μm, and m is the slope of the particle size-number distribution. This index has an advantage over the BP limit tests in that it is not affected by the type of instrumental principle used to determine the parameters of the size distribution (see the sections, "Electronic Particle Counters" and "Comparison of Microscopic and Electronic Particle-Counting Methods"). In addition, C can be calculated from data acquired at size thresholds that do not necessarily coincide with those of the BP tests, and calculations of C can be made readily and routinely using on-line computerized quality control procedures.

The background of the establishment of particulate matter standards for small-volume injections is reviewed in another later section.

VISUAL INSPECTION: MANUAL METHODS

Manual inspection by human inspectors for the presence of visible particulate matter in parenteral solutions still remains the standard, in-line, 100% inspection method. While elec-

tronic television monitors have made significant strides in replacing 100% human inspection, the former remains standard practice for end-product particle analysis of parenteral products.

Each final container of a parenteral product must be inspected by a trained individual. Any evidence of visible particulate matter or other product/container defect provides the grounds for rejecting that container.

Task Group No. 3 of the Parenteral Drug Association published guidelines to be considered in the design and evaluation of visual inspection procedures (38). These guidelines are discussed in this section.

Equipment

Lighting may be fluorescent, incandescent, spot, and/or polarized. The most common source of light is fluorescent. The light source may be positioned above, below, or behind the units being inspected. The range of light intensity may vary between 100 and 350 foot-candles.* This intensity can be achieved either with one 100-watt, inside-frosted incandescent lightbulb or with three 15-watt fluorescent bulbs with the container held 10 inches from the light source. Certain types of products (e.g., colored solutions) or certain types of containers (e.g., amber) require increased light intensity compared to that normally used. As light intensity begins to weaken due to age of the bulbs or usage, lamps should be replaced. Good practice demands that inspection lamps be monitored periodically.

A white-and-black background lighted with nonglaring light is the standard environment used for visual inspection of product containers. The white background aids in the detection of dark-colored particles. Light or refractile particles will appear against the black background.

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* The Japanese Pharmacopeia (JP) reqires 740–930 foot-candle light intensity for inspection of injections contained in plastic containers (39).

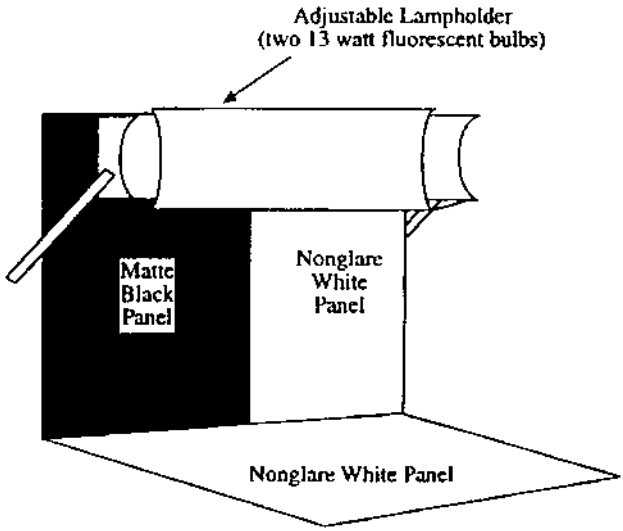

Fig. 3.1 Apparatus for visible particle inspection as presented in the European Pharmacopeia, Figure 2.9.20.-1, p. 222.

The EP provides a figure of the type of apparatus to be used in visible inspection of particulates (3) (Fig. 3.1). The apparatus consists of

a matte black panel of appropriate size held in a vertical position
a nonglare white panel of appropriate size held in a vertical position next to the black panel
an adjustable lampholder fitted with a suitable, shaded, white-light source, a suitable light diffuser (details provided in the EP), and illumination intensity maintained between 2000 lux and 3750 lux, with higher intensities preferred for colored glass and plastic containers

Many manufacturers have progressed to the use of automated inspection equipment. Appropriate inspection procedures should specify and monitor the adjustment of the machine's operating parameters required to achieve a quality

inspection that is at least equivalent to that resulting from a previously established manual inspection procedure. Automated inspection machinery is discussed in the section, "Visual Inspection: Automatic Methods."

A standard inspection booth contains an all-black interior except for the front entrance for the inspector. A vertical screen in the back of the booth is half black and half white. Light usually is projected vertically with frontal blockage to protect the observer's eyes from direct illumination. A magnifying lens at 2.5 × magnification may be set at eye level to aid the inspector in viewing the container in front of the white/black background. Excellent viewing is provided without distraction, and acuteness of vision is increased to improve the level of discrimination. It could be argued that the level of discrimination becomes too high; that is, containers are rejected that would not have been rejected had no magnification been used.

Inspection cabinets should have black side walls with a baffle to prevent the light source from impinging on the inspector's eye. Fluorescent lamps provide a better light source because they are more diffuse than incandescent lamps.

Methodology

Most inspection processes are referred to as off-line inspections, in which the inspection procedure occurs at the completion of the manufacturing, filling, and sealing process. In-line inspection of container components can also be done, especially if the production process can be suitably adapted to achieve the desired results without increasing the risk of microbial and particulate contamination. Obviously, the removal of defective containers, such as those showing cracks or the presence of particles, prior to the filling of the product ensures product quality and minimizes loss of expensive drug products.

Standard operating procedures for inspection of parenteral containers depend on the kind of container inspected;

Table 3.4 Basic Procedure for Manually Inspecting Clear Solutions for Visible Evidence of Particulate Matter

1.	Container of parenteral solution must be free of attached labels and thoroughly cleaned. Use a dampened, nonlinting cloth or sponge to remove external particles.
2.	Hold container by its top and carefully swirl contents by rotating the wrist to start contents of the container moving in a circular motion. Vigorous swirling will create air bubbles, which should be avoided. Air bubbles will rise to the surface of the liquid; this helps to differentiate them from particular matter.
3.	Hold the container horizontally about 4 inches below the light source against a white-and-black background. Light should be directed away from the eyes of the inspector, and hands should be kept from under the light source to prevent glare.
4.	If no particles are seen, invert the container slowly and observe for heavy particles that may not have been suspended by swirling.
5.	Observation should last for about 5 seconds each of the black-and-white background (24).
6.	Reject any container having visible particles at any time during the inspection process.

that is, procedures will be slightly different for ampules than for large-volume glass bottles, for amber vials than for flint vials, and for plastic bags than for glass containers. However, a basic procedure can be followed regardless of the type or size of container; an example of such a procedure is given in Table 3.4.

Personnel

The human inspector determines the quality and success of the manual inspection process. Since the inspection process is subjective in nature, the main limitation of the process lies with restriction in the vision, attitude, and training of the individual inspector.

As a minimum standard, personnel assigned as inspectors should have good vision, corrected, if necessary, to ac-

ceptble standards. Inspectors should not be color-blind. Visual acuity should be tested at least on an annual basis.

Good attitude and concentration cannot be overemphasized. One of the major limitations of human inspection for particulate matter is reduced efficiency of the individual because of a lack of concentration. This can easily occur if the inspector suffers from extreme worry or other distraction from outside personal pressures. Obviously, emotional stability is an important criterion in selecting inspectors.

Fatigue also becomes a major limitation of human inspection. Personnel should be provided appropriate relief from the inspection function by rotating jobs and allowing for rest periods.

Formal training programs must precede the acceptance of an individual as a qualified inspector. The training program should include samples of both acceptable and unacceptable product containers that must be distinguished by the trainee. During the training period, all units inspected by the trainee should be reinspected by qualified inspectors to ensure the quality of the inspection and the development of the trainee. After the inspector has passed the training period, performance tests should be done at random intervals to ensure that quality standards are being maintained.

Two reports have been published concerning the effect of personnel experience on detection of particles in ampules. Graham et al. (40) found that inspectors with no experience and inspectors having at least 10 years experience agreed 64% to 83% of the time regarding ampules inspected under various conditions. Experienced inspectors were faster in the inspection process. Baldwin et al. (41) found that experienced inspectors reject ampules at a greater rate (28.3%) than did nonexperienced inspectors (13.2%). Discrimination in particle detection apparently correlates with training and experience.

Acceptance Standards

Visible evidence of particulate matter in parenteral products, both solids and liquids, is considered by most parenteral prod-

uct manufacturers to be a very serious (critical) defect. Therefore, acceptable quality levels (AQLs, the highest percentage of defective units unacceptable for releasing the batch) for statistical sorting samples taken for particulate and other quality inspection generally are within the range of 0.25% to 1.0%. For example, with a sample of 315 vials and an AQL of 0.25%, finding 2 vials with visible foreign particulates will be acceptable, but finding 3 vials with particulates will cause rejection or resorting of the batch.

Japanese Method for Inspection and Analysis of Particulate Matter

Requirements for freedom of parenteral solutions from the presence of particulate matter are very strict in Japan. Inspection of individual containers for any visible evidence of particulate matter is done much more rigorously. For example, an inspector in a typical Japanese pharmaceutical company will take up to 10 seconds inspecting a single vial of a parenteral solution. Contrast this with inspectors in a typical fast-speed American parenteral manufacturer; they will inspect 50–150 vials per minute for evidence of particulate matter.

The Japanese technique for preparation and testing of solutions for the presence of particulate matter by microscopic analysis deserves some attention. The meticulousness of their preparation techniques are impressive. For example, all materials (forceps, petri dishes, filtration funnels) used in filtering solutions are first sonicated for at least 5 minutes, then washed thoroughly with particle-free water three times. The membrane filters used for the blank controls are washed thoroughly using a very rigid procedure involving starting at the top of the nongridded side of the filter, sweeping a stream of particle-free water back and forth from top to bottom, then repeating this on the gridded side of the filter. After inserting the filter into the filter holder base and installing the funnel, the entire system is rinsed twice with particle-free water, taking care not to allow the rinsings to pass through the filter. Further rinsings are completed with the water vacuumed

through the filter. Interestingly, this water is introduced into the funnel using an injection syringe fitted with a 0.45-μm filter. The maximum allowable number of particles for the entire membrane filter pad used as the blank control is 3 that are 10 μm or longer and 1 that is 50 μm or larger using a suitable microscope with 40× and 100× magnification with incident light at an angle of 20°.

Sample test solutions are handled in the same way. Five vials are filtered through the same filter pad. Some Japanese companies even use a filter pad that is only 4 mm in diameter. Filter pads, after vial contents have been filtered through the pads, are photomicrographed, usually at the 40× magnification. Test results are judged by visual comparison of the test filter pads with reference photographs of previous test samples judged by the Quality Control Department to represent the particulate quality desirable with the product sample.

Comparison to Other Particle Inspection Methods

Manual visual inspection often is criticized because of its apparent inconsistency and unreliability. Its subjective nature, depending on the judgment of uncontrolled and variable human evaluation of what may or may not be seen, drives many quality control specialists to seek other methods for achieving the same purpose—100% nondestructive inspection of parenteral products for the presence of particulate matter.

Manual inspection can be compared to automatic electronic inspection methods on the basis of precision and accuracy (42). Precision is related to *consistency*, which measures the capability of any given process to detect the same conditions in repeated blind tests. *Accuracy* relates to bias, based on inequality of reject rates. When the precision and accuracy of manual inspection were compared to those demonstrated by Autoskan, an electronic video particulate inspection machine (see the section, "Autoskan System"), two interesting conclusions were drawn:

1. Consistency of both methods, based on Cohen's kappa statistic, was comparable. Autoskan was not superior to man-

ual inspection in terms of repeatedly rejecting those ampules containing particles.

2. Based on Cochran's Q statistic, bias was a problem with human inspection, while appropriate settings of the Autoskan could eliminate machine bias. However, rejection rates were established using only one machine, while eight inspectors were tested and compared for their rejection rates for the same batch of ampule products. An example of rejection rates comparing machine and human inspectors is given in Table 3.5.

Reproducibility in human visual inspection was the subject of an article by Faesen (43). Each of 1000 diamond-numbered ampules and vials was inspected by 10 different inspectors, twice for each operator, using a Liquid Viewer, an inspection cabinet, and a Rota Ampul Inspection Machine (for ampules only). The total number of rejects was registered following 60 inspections for the 1000 ampules (three inspection methods performed twice) and 40 inspections for the 1000 vials (two inspection methods performed twice). Results indicated that reproducibility in a visual inspection was nearly twice as high when performed with a Liquid Viewer as compared with reproducibility for inspections performed with the inspection cabinet. For ampules, the value reached using the Ampul Inspection Machine was less than 10% units higher

Table 3.5 Reject Rates (%) for Four Products Inspected for Particulate Matter by Autoskan and Eight Human Inspectors

		Eight human inspectors	
Product	Autoskan	Average	Range
A	22.2	25.2	19.7–29.2
B	20.2	21.5	17.5–24.0
C	20.3	17.4	15.5–19.5
D	7.2	7.9	5.6–10.0

Source: Ref. 42.

than that with the inspection cabinet. The Liquid Viewer appeared to be the superior instrument for visually inspecting parenteral solutions.

In a panel discussion of mechanical inspection of ampules (44–46), it was stated that the average reject rate for ampules inspected manually was 2–2.5%. Three inspection machines were compared to manual inspection. Autoskan equipment (44) showed a reject rate of 14.1%, the Rota Machine (45) increased the reject rate 0.5–1.5% over that for manual inspection, and the Strunck Machine (46) yielded a reject rate of 2.98%. The major advantage of machine inspection was simply the substantial increase in the number of containers inspected per unit time.

Blanchard et al. (47) compared the human visual examination method with several other methods for detection of particulate matter in large-volume parenteral solutions. Visual methods using either the naked eye under diffuse light or a 2.5 opter lens under diffuse light proved to be inadequate to other methods (light scattering, Prototron, and microscopic examination after filtration) in terms of sensitivity to low levels of particulate contamination. Not surprisingly, the visual method showed a high degree of subjectivity.

Visible Particle Sizes to the Unaided Eye

Since the number and size of particles in parenteral solutions have become important characteristics to evaluate, it has been assumed that particles larger than 40 or 50 μm are detectable by the unaided eye. Thus, in complying with USP requirements that any container showing visible evidence of particulate matter be rejected, it must be assumed that the average inspector will pass those solutions containing particles with a size of 40 μm or less. This, of course, presents some discomfort for those who believe that particulate matter, especially in the size range of 10–40 μm, is clinically hazardous.

It is not only the size, but also, and probably more importantly, the number of large particles injected into man intrave-

nously that is considered dangerous. Thus, official standards have been enforced for maximum allowable numbers of certain size particles in parenteral solutions.

At least one attempt has been made to quantify the size and concentration of particles that can be detected by the unaided eye (8). In a standard booth, 5 ml ampules containing 10 to 500 particles per milliliter of particle sizes between 5 and 40 μm (using polystyrene beads) were inspected by 17 inspectors. Based on a multiple linear analysis model that calculated the probability of rejecting an ampule as a function of particle size and concentration, sizes of particles detected at various concentration levels at 50% and 100% probability of rejection rates were predicted. These data are reproduced in Table 3.6. The authors concluded that a 50% probability of rejection rate be achieved with 20-μm particles in sample solutions in order for potential inspectors to be qualified for in-line inspection. However, it is interesting to note that a minimum

Table 3.6 Size of Particles of Varying Probability Levels[a]

Particle concentration	Particle size (μm), 50% chance	Particle size (μm), 100% chance
USP limit 50 particles/ml[b]	18.82	51.45
USP limit 5 particles/ml[c]	19.96	54.88
1-ml ampule, 1 particle	20.07	55.21
2-ml ampule, 1 particle	20.08	55.25
5-ml ampule, 1 particle	20.09	55.28
10-ml ampule, 1 particle	20.10	55.29
20-ml ampule, 1 particle	20.10	55.29
50-ml vial, 1 particle	20.10	55.29
1-liter large volume, 1 particle	20.10	55.29

Source: Ref. 8.

[a] Arcsin $\sqrt{P_1} = 0.33689252 + 0.02231515$ size $+ 0.000035$ size versus concentration -0.00008694 concentration.

[b] Not more than 50 particles/ml equal to or larger than 10 μm.

[c] Not more than 5 particles/ml equal to or larger than 10 μm.

particle size of 55 μm was required for all inspectors to reject all solutions containing this size of particle.

VISUAL INSPECTION: AUTOMATIC METHODS

Introduction

Manual visual inspection continues to be the most commonly used quality control method for particle detection in parenteral products. The limitations of depending on human inspection for rejecting particle-contaminated solutions have already been addressed. High technology strives for sophisticated automatic methodology to replace the dependency on human manual inspection. One area of high-technology application to particle analysis in parenteral products is the development and improvement of electronic particle counters. The main limitation in the use of these instruments in particulate matter analysis resides in the fact that the tests are destructive. For each final container of parenteral product, 100% inspection cannot be accomplished with electronic particle counters. The same limitation holds true for automated microscopic methods. The area of technology that offers the greatest potential for replacing human examination in 100% container inspection requirements is the area of computer-controlled, automatic electro-optic systems. Such systems are rapid, nondestructive, and reproducible in their inspection of parenteral products for foreign matter.

Early attempts to automate the 100% inspection process were reviewed by Groves (24). Systems developed and tested included the Brevetti device (48), Strunck machine (49), and the RCA machine (50). Despite considerable electronic ingenuity, all of these systems required human intervention at some stage of the inspection process, although Groves admitted this may be a consolation.

Hamlin et al. (51) were among the first investigators to test the use of television (TV) as an inspection device in detecting subvisible particulate matter. However, their main emphasis in using TV monitoring was as a research tool in

detecting 10-μm particles in experimental formulations for prediction of estimated shelf life based on physical stability. Also, TV monitoring required human involvement in viewing and rejecting particle-contaminated solutions.

Technology has made significant improvements in fully automated parenteral product inspection procedures. Disadvantages of earlier automated systems, such as lack of standardization of performance, separating marks on the outer container surface from particles inside, failures to detect underfills or empty containers, and machine variabilities, have largely been eliminated with the automated systems available today.

Video inspection employs one of two basic mechanisms for automated container inspection (52). One mechanism uses imaging optics in which the particles suspended in the solution are illuminated by a fiber-optic light system and imaged on a video display. The other mechanism employed in automated video inspection is based on light scattering from particulate matter, which is then received by a detection system and projected onto a television camera system. Several systems commercially available employ the light-scattering principle for automated video inspection. Among the most widely used systems are the Autoskan system, the Eisai Ampul Inspection Machine (AIM) system, Particulate Detection System Ampules and Vials and the Schering PDS/A-V system. The Prototron system (53,54) at one time was a widely used nondestructive inspection method using laser light, but it is no longer used today.

Autoskan System

The Autoskan system uses white light—in contrast to laser light, which was used by the Prototron system—to illuminate particles suspended in parenteral solutions. Particles will scatter the light, which is received by a television camera system. Any solution that contains particles will generate an error signal, and that product container will not be released by the Au-

toskan system at the accept station. Containers are also automatically rejected if they are either underfilled or overfilled.

Autoskan became the first totally automatic inspection system developed to detect particulate matter in injectable solutions. The instrument is suitable for the inspection of vials, ampules, cartridges, and syringes. In Fig. 3.2, ampules on a rotary feed table are fed into the turret. The turret picks up the ampules and intermittently transports them around to the inspection station, where the lens of the television camera is located. The ampule is magnified by high-intensity light from below the check holding the ampule (see Figure 3.3). This light reflects particles moving in the liquid, making them visible to the camera (often also visible to the human eye). The Autoskan checks in the turret contain motors that spin each con-

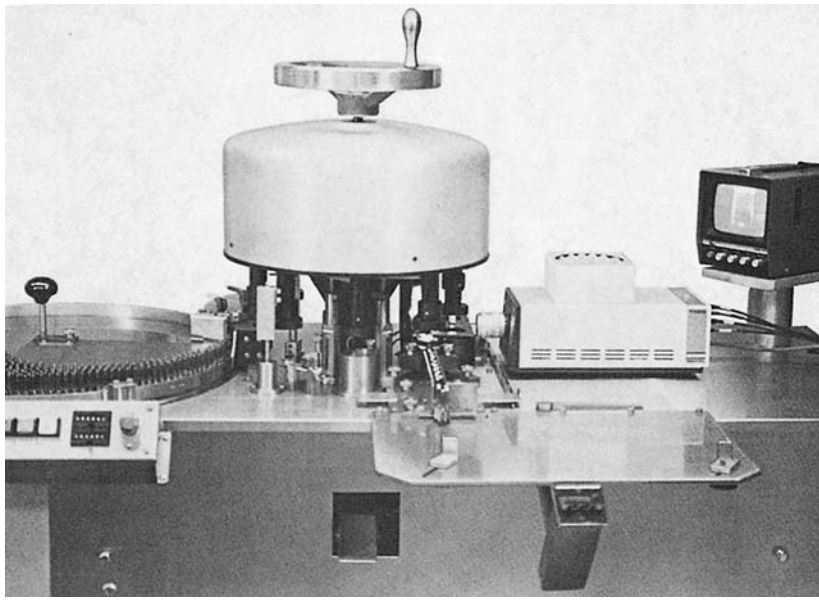

Fig. 3.2 Autoskan inspection system showing ampules on a rotary feed table leading into and exiting from the detection area. (Courtesy Lasko Company, Leominister, Pennsylvania.)

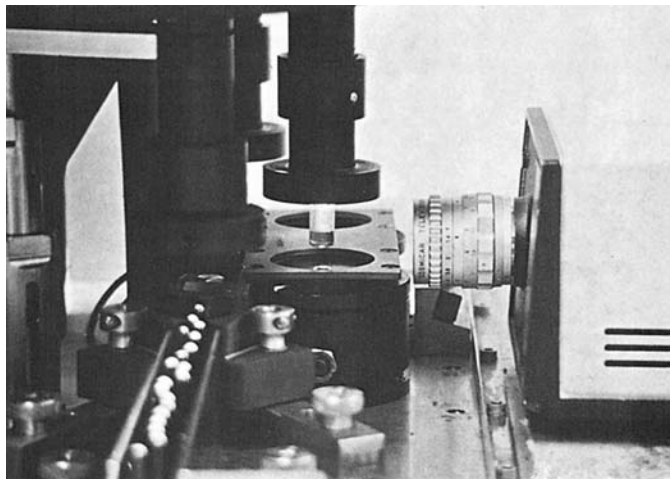

Fig. 3.3 Close-up of ampule being inspected for particles by the Autoskan system. (Courtesy Lasko Company, Leominster, Pennsylvania.)

tainer at an adjustable speed until the container comes before the lens of the television camera. The spinning is designed to dislodge and set particles in motion and create a central vortex in the liquid. This permits the television and electronic system to detect underfilled, overfilled, or empty containers. The inspection area of the container is preset. Liquid levels that do not exactly fit within the upper and lower limits of the inspection area are rejected automatically. If the container has the correct fill volume, it then "becomes eligible" for the inspection process that detects the presence of foreign matter.

A "master picture" of the correctly filled container is taken simultaneously with the liquid level pictures. The master is put into Autoskan's electronic memory, which serves as a standard for subsequent comparative video images of the same container. Sixteen comparison pictures of the container are taken and compared to the master picture. Any difference between the master and any of the subsequent comparitive

pictures of the single container will result in that container being rejected. Since the Autoskan has the capability of inspecting between 1800 and 4500 containers per hour, the time for checking the liquid level, taking a master picture, and subsequent comparison pictures is less than 1 second per container.

The fact that the liquid contents are swirling while the container itself is motionless during the inspection process has a very important implication. The master picture is based on a motionless container. All scratches, printing, or other marks on either the outer or the inner surface of the container are part of the master picture. Any difference between the master and any one of the subsequent comparison pictures of the single container, therefore, would be caused only by particulate matter moving within the liquid contents, reflecting light back to the camera.

Louer et al. (55) compared Autoskan's performance against visual inspection for discrimination of "good" and "bad" ampule solutions in terms of particulate contamination. Their results, reproduced in Table 3.7, showed that, on the average, there was a 93% rejection of control bad ampules by the machine, whereas the percentage for visual inspection was

Table 3.7 Comparison of Visual Inspection Versus Autoskan Automatic Inspection of Ampules for Particulate Matter

	Visual inspection, 50 examinations (average)	Machine inspection, 10 runs
Ampules (n = 50) rejected from the control bad lot	26.6	42.7 of 46
Percentage of bad lot rejected	53.2	92.8
Ampules rejected from the control good lot	3.16	39.6 of 752
Percentage of good lot rejected	0.42	5.25

Source: Ref. 55.

only 54%. The rejection of good ampules by visual inspection was significantly lower than the rejection of good ampules by the machine.

Eisai Ampul Inspection Machine System

Like the Autoskan system, the Eisai AIM system uses white light as the source of detection of particles. However, whereas Autoskan measures light scattered from a particle, Eisai detects the moving shadows produced by foreign matter in a container of solution. As with the Autoskan, each container is spun around and stopped so that only the liquid in the container is still rotating when the container enters the light. If any foreign matter is floating and rotating in the liquid, the light transmitted through the liquid is blocked, and a shadow is cast by the moving particles. Eisai systems employ a phototransistor that converts moving shadows into electrical signals. These signals are compared to preset detection sensitivity signal standards, and if the standard sensitivity is exceeded, the container is rejected. Like the Autoskan, the Eisai detector does not react to scratches, stains, colors of the ampule, or the color of the liquid contents since these are all perceived as stationary objects.

The Eisai system, like the Autoskan system, checks the volume of liquid in the container and can reject overfilled, underfilled, and empty containers. The shadow cast by the liquid meniscus of a properly filled container is expected to fall within a certain preset range within the inspection field. If it falls above or below this range, the container is rejected. Adjustments in the Eisai system can be easily made for different ampule sizes, ampule color, and viscosity of the liquid contents.

The conveyance-and-inspection mechanism of the Eisai system is shown in Fig. 3.4. Ampules are conveyed by the star wheel onto the inspection table, spun at a high speed, and stopped before reaching the light beam. When the ampule enters the light beam, the light projector and detector follow the

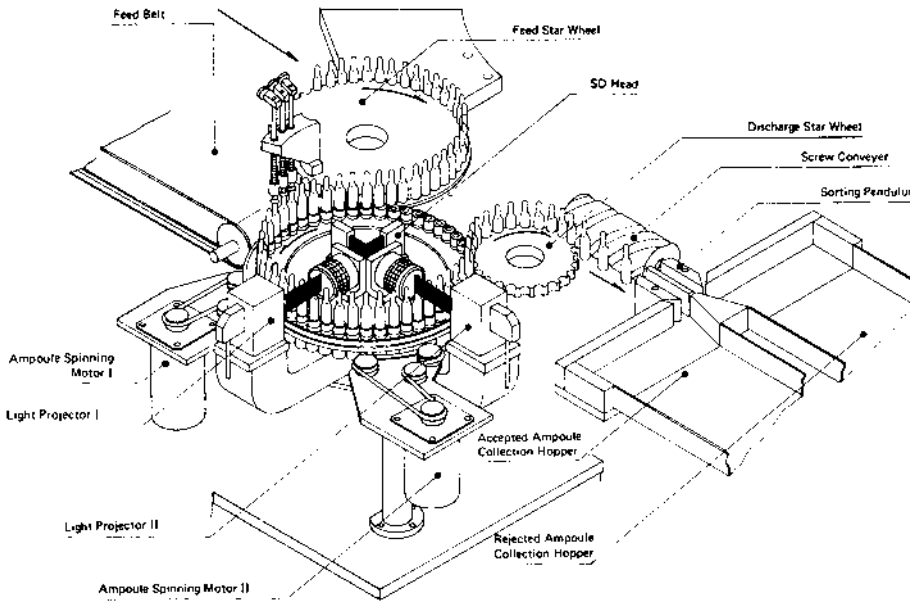

Fig. 3.4 Conveyance and inspection mechanism of the EISAI automatic inspection system. (Courtesy Eisai, USA, Inc., Torrance, California.)

ampule while liquid is still rotating inside. After one ampule is inspected by two sets of projectors and receptors (thus, a double-inspection system), the next ampule is carried through the same process. Ampules are moved by the screw conveyor to the sorting pendulum, where rejected and accepted ampules are separated. The AIM system automatically keeps count of the number of accepted and rejected containers and displays these numbers on the display panel.

Performance evaluations of the Eisai AIM system have been conducted by at least three major pharmaceutical companies: Pharmacia (now Pfizer), Organon, and Merck. The complete report of these evaluations is available from Eisai USA, Incorporated. One evaluation was reported at the 1982 annual meeting of the Parenteral Drug Association. All three investi-

gators concluded that the inspection of ampules by the AIM system compared to manual inspection resulted in a great improvement in the quality of ampules accepted and released. Using the performance criteria model published by Knapp et al. (discussed in the section, "Probabilistic Particulate Detection Model"), Upjohn found that the Eisai machine will do a better job than manual inspection in rejecting defective ampules for production lots. Organon found the Eisai machine to be more reproducible than human inspection as long as the product in the containers was not an oil or had the tendency to foam. Merck found that the quality of the Eisai-inspected material was more than twice as good as the quality of those containers manually inspected. Advantages of the Eisai system itemized by the Merck report include (1) versatility, that is, ability to handle a large variety of ampule and vial sizes, products, and viscosities; (2) adjustable sensitivity level; (3) attainable speeds; (4) results of performance studies; and (5) price.

To our knowledge, there is no published report directly comparing the inspection performances of Autoskan and Eisai.

Schering PDS/A-V System

Schering Corporation has patented the PDS/A-V, a fully automated particulate inspection system (56). A photograph of the system is shown in Fig. 3.5.

Containers are conveyor fed from oriented trays into the inspection star wheel. Light is directed into a container using fused fiber-optic pipes formed into a narrow slit. The container is spun, creating motion of particles in the liquid inside. The entire container is scanned by a fiber-optic image dissector, which forms multiple-image planes of the entire liquid volume. The image dissector transmits light scattered from moving particles in the container to a set of matched photodiodes, where the light is changed into an electrical signal and processed. Only signals from moving particles are processed;

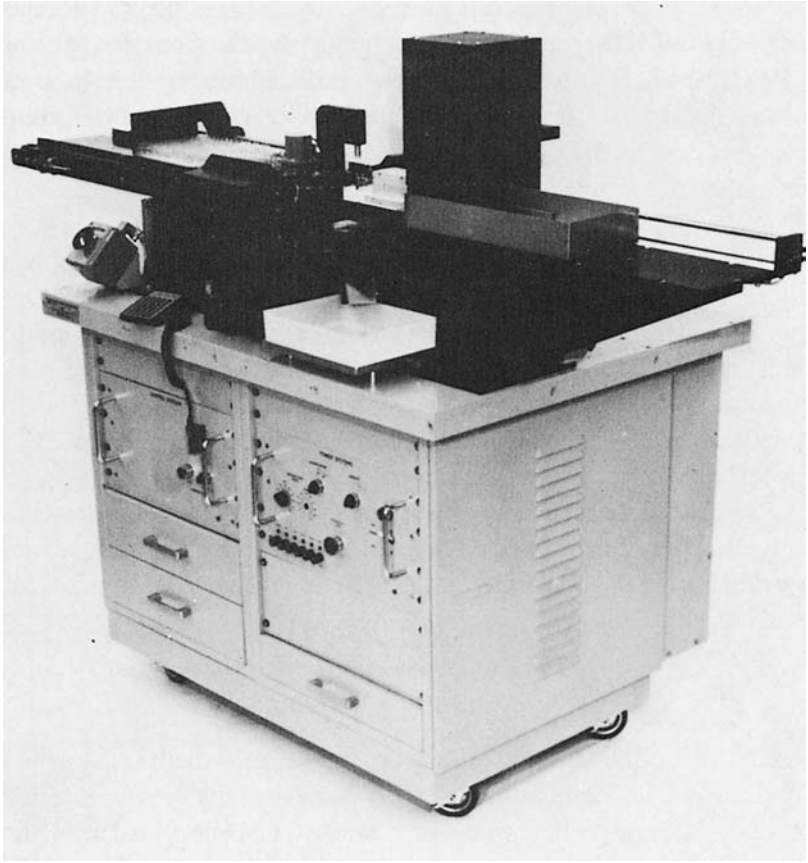

Fig. 3.5 Particulate Detection System 100 for ampules and vials. (Courtesy Electro-Nucleonics, Inc., Fairfield, New Jersey.)

thus, container defects or printing do not generate false rejects. The image dissector inspects first the lighted lower part of the container for glass particles, then the full volume of the container for other particles, including those floating at the meniscus. Containers are rejected by a single-board microcomputer if the scattered light detected results in a higher score than the digital rejection criteria stored in the computer. The device can inspect 10,000 containers per hour. More elaborate

details of the successful automated inspection device are given in articles published by Knapp et al. (56–61).

Probabilistic Particulate Detection Model

Knapp and coworkers published a series of articles describing the theory and application of a probabilistic inspection model in the automated nondescriptive particulate analysis of sealed parental containers. In his most recent publication (57), Knapp compares the application of his probabilistic model and statistically defined particle contamination quality regions to the application of the probabilistic concepts developed for heat sterilization validation (e.g., Fo) with respect to improving product quality.

The probabilistic model is based on the finding that particulate inspection methodologies, human or robotic, are probabilistic rather than deterministic in nature (58). In other words, no final container of solution is acceptable or unacceptable; rather, each final container of solution possesses a probability of being rejected for whatever inspection process is being evaluated. Rejection probabilities are determined simply by recording the number of times a numbered container is passed and the number of times that same container is rejected during a manual or automatic inspection process. Each container accumulates an accept/reject record. If 1000 containers are inspected several times and each of the 1000 containers yields an accept/reject ratio, a histogram can be constructed plotting the number of containers in each probability group against an empirically determined rejection probability. Such a histogram is shown in Fig. 3.6, and it represents the cornerstone for the conversion by Knapp et al. of particulate inspection from a craft to a science (61).

The abscissa in Fig. 3.6 represents rejection probabilities grouped arbitrarily into 11 intervals. The ordinate represents the logarithmic number of containers (vials) within each of the 11 probability groups. For example, of the 1000 vials inspected for particulate contamination, 805 vials were found to be par-

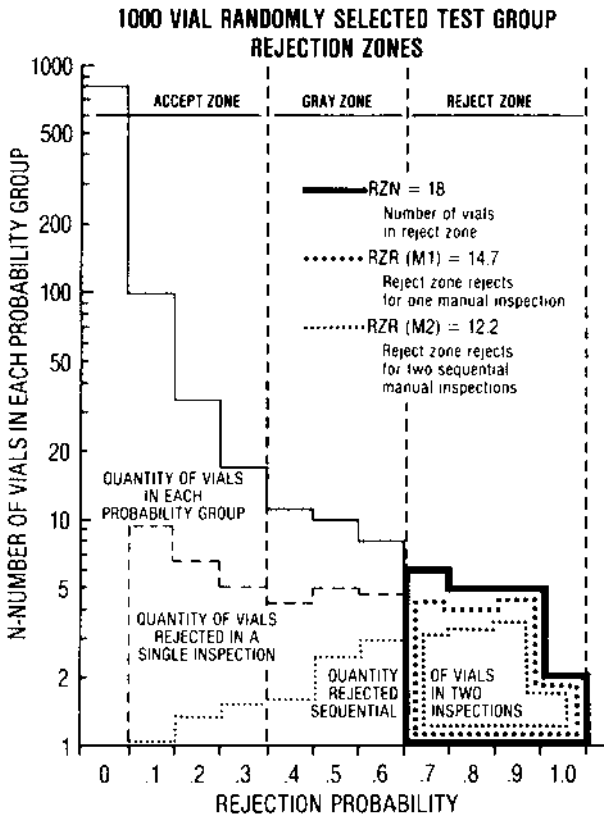

Fig. 3.6 Histogram plotting number of vials per each probability of rejection group. (Courtesy J. Z. Knapp, Schering Corporation, Kenilworth, New Jersey.)

ticulated free in each of the 50 inspections, while 2 vials contained particulates that were detected in each of the 50 inspections.

The dashed lines on the lower half of the histogram show the average number of vials rejected in a single inspection or two sequential inspections in each probability group. These values are obtained from the relationship (58)

$$P(Mn)_i = P(MI)_i$$

where $P(Mn)_i$ is the rejection probability associated with the nth manual inspection in a probability group, $P(MI)_i$ is the quality of vials rejected in a rejection probability group in a single inspection, and n is the number of inspections of rejected material. For example, of the eight vials located in the 0.6 rejection probability $P(MI)_i$ group, five were rejected following a single inspection, while only three were rejected following two sequential inspections. This indicates that improved discrimination occurs following a reinspection of initial rejects. The reinspection was utilized as a practical response to the existence of particulates even in well-controlled parenteral manufacturing areas below the range of present medical and FDA interest (59). From the information contained in the reinspection histogram of Fig. 3.6, Knapp and Kushner (58) defined three zones within the rejection limits of 0 and 1.

The accept zone contains all vials that have less than 1 chance in 10 of rejection in two sequential inspections. The reject zones contain all vials that have at least 1 chance in 2 of being rejected in two sequential inspections. The gray zone exists between the accept and reject zones. For single inspections, the probability limits for the three zones are seen in Fig. 3.6, where

Accept zone, $p \leq .3$
Gray zone, $.3 \leq p \leq .7$
Reject zone, $p \geq .7$

Figure 3.6 also shows three terms: RZN, RZR (M1), and RZR (M2). The definitions of these terms are given in the figure. Their calculations are explained thoroughly in Ref. 58. Using these terms, a variety of parameters can be measured, including reject zone efficiency (RZE) and undesired reject rate (RAG). By definition, RZE = RZR/RZN. In the example in Fig. 3.6, the RZE after a single inspection is 81.7%. This means an 81.7% probability exists for a manual single-inspection method to reject those vials known to exist in the reject zone. Matching or exceeding this objective measure of the security achieved by a manual parenteral inspection procedure

should be the only good manufacturing practice (GMP) requirement for validation of any alternative inspection technique or process (58).

The availability of the probabilistic model for particle inspection of sterile product solutions in their containers has permitted objective evaluations of various inspection parameters, new methodologies, and new detection equipment. For example, the Schering Particulate Detection System for Ampuls (PDS/A) was validated using the probabilistic methodology (56,59). RZE scores were used to determine the effects of lighting levels, light polarization, and lens magnification on a human inspection of vials mechanically positioned by an experimental machine at Upjohn (now Pfizer) (62). RZE scores permitted the selection of optimal settings for light, magnification, and light polarization. Interestingly, however, RZE scores also showed that the mechanical handler was not as efficient in meeting the minimum Upjohn standards for performance as their currently used inspection process. The probabilistic model allowed a valid decision to be made based on objective scientific data.

Knapp concludes his most recent publication (57) by stating that this methodology is now used worldwide and is being applied to define the performance of most automated particle inspection systems.

CURRENT ISSUES WITH VISIBLE PARTICLE DETECTION AND INSPECTION PROCEDURES

Inspection of solutions for visible particulates has taken on new priority and scrutiny in the past several years. Part of this is because of increased FDA and other regulatory compliance groups demanding higher degrees of product quality with respect to visible particulates. Another part is the struggle of the industry to define quality policies related to visible particulates in light of the dichotomy that, although everyone accepts the fact that particulates can and do exist in solutions (i.e., you will never see a solution with zero particulates), we

are supposed to approve and release only those products that supposedly have no particulates. Where do you draw the line with respect to acceptable or unacceptable product quality?

There are significant struggles with interpreting the pharmacopeias with respect to visible particle testing for parenterals.

All sterile products are subjected to a 100% inspection, typically automated to some degree. However, each unit of product is viewed/analyzed by human inspection for only a second or two (at most maybe 5 seconds, with the exception of Japan, which may inspect for up to 10 seconds per unit of product). Following 100% inspection, product lots are subjected to a more demanding release test, described in the compendia. In fact, the compendia have the following definitions:

USP: "essentially free from particles that can be observed on visual inspection"
EP: "clear and practically free from particles"
JP: "clear and free from readily detectable foreign insoluble matter"

These compendial requirements plus current industry practices with respect to visual inspection beg the following questions (63):

1. How are 100% on-line inspections separated from release testing when on-line inspections require rejection of all product units that contain particles while release testing requires that product units "be essentially free from particles"?
2. How are the compendial requirements "essentially free from particles" to be interpreted?
3. Is there any possibility that inspection methodology for visual particulates will be hormonized worldwide?
4. Is there any possibility that "haze" testing can be harmonized and acceptable to all compendia?

FDA presentations have indicated that quality control of visible particulate matter is of great concern to inspectors. A

review of Warning Letters issued in 1999 and 2000 (64) from the FDA to manufacturers of injectable products provides the most often cited specific observations with respect to concerns about visible particulates:

1. For the same lot, there is sometimes a high rejection rate by certain personnel, while other personnel have low rejection rates.
2. The results from statistical sampling at the beginning, middle, and end of a filling operation found no defects, whereas the entire lot was rejected for release because of visual defects during 100% inspection.
3. Failure to establish a maximum acceptable level of vials rejected during 100% inspection.
4. Failure to take adequate action to ensure the quality of released product when the particulate reject limit was exceeded.

Personnel responsible for detection of visible particulate matter must be thoroughly trained for this important quality evaluation. Training is not an easy task because of a variety of reasons: vision capabilities, concentration, sample standards of particulate types, inspection environment, and qualifications of the trainer(s).

USP TEST FOR SUBVISIBLE PARTICULATE MATTER IN LARGE-VOLUME INJECTIONS FOR SINGLE-DOSE INFUSION AND SMALL-VOLUME INJECTIONS

After several years of collaborative effort among laboratories from the FDA, universities, and pharmaceutical manufacturers, a method became official in the First Supplement of the USP (19th edition) in 1975 for the particulate matter analysis and release specifications for single-dose large-volume parenterals (LVP now LVI). The original method involved the filtration of 25 ml of solution through an ultraclean membrane filtration assembly, then observing the membrane and counting entrapped particles on its surface under a microscope using 100× magnification.

Since the advent of the microscopic test for LVI solutions, the particle load in these solutions was substantially reduced. This was recognized in the early 1980s such that attention turned to establishing subvisible particle standards for small-volume injections (SVIs). By the USP XXI (July 1985), the electronic light obscuration (LO) test method was introduced for SVIs. Until around 2000, LVIs were evaluated for subvisible particulate matter using the microscopic method, while SVIs were primarily evaluated by the light obscuration method. However, effective with the USP XXV, both LVI and SVI solutions are now primarily evaluated for the presence of subvisible particulates by the light obscuration method. If the light obscuration method cannot be used, then the microscopic method is allowed. Many companies perform both methods in measuring the amounts and types of particulate matter in their parenteral solutions and reconstituted powders. Also, effective with the USP XXV, both LVIs and SVIs followed the same general USP guidelines (test apparatus, calibration, test environment, test procedures, and calculations) for both the light obscuration particle count test (pp. 2046–2052) and the microscopic particle count test (pp. 2046–2052).

Development of the Small-Volume Injection Subvisible Particle Test

Back in the early 1980s, there was a general consensus that these LVI limits were too strict for SVI solutions and, in fact, SVIs should not have particle limits because (a) volumes administered are much smaller than those for LVIs, and (b) health hazards from injected particulates were not unequivocally established. Nevertheless, the USP sponsored studies to establish particle limits for SVI solutions reasonable for both a safety standpoint and a quality control standpoint achievable by the parenteral industry.

Two SVI particle limit proposals were published in late 1983 (65). One was based on particles per container proposed by the USP Subcommittee on Parenteral Products. The other

proposal, by the USP Panel on Sterile Products, was based on particles per milliliter. A comparison of the two proposals is summarized below:

	≥25 μm	≥10 μm
Subcommittee proposal	1000 Particles per container	10,000 Particles per container
Panel on sterile products proposal	70 Particles per milliliter	250 Particles per milliliter

The subcommittee proposed limits based on the following rationale: The addition of up to five containers of any SVI to a 1-liter LVI solution should not increase the number of particles by more than double those allowed by the USP limit for LVI solutions (5 particles per milliliter ≥ 25 μm or 5000 particles in 1 liter ≥ 25 μm; 50 particles per milliliter ≥ 10 μm or 50,000 particles in 1 liter ≥ 10 μm). If five additives, each containing no more than 1000 particles per container ≥ 25 μm, were admixed with the 1 liter LVI containing 5000 particles ≥ 25 μm, the total particles 25 μm or larger would be 10,000, which would be the maximum allowable particle number per admixed solution. At 10 μm, the total particle number with five additives in a 1-liter LVI would be 100,000, which would be no more than double that of the LVI alone. Therefore, the subcommittee proposal was based on concern more for the cumulative particulate insult the patient might receive than for the number of particles per milliliter of solution.

The USP panel proposal was based on data from an FDA survey of a large number of small-volume parenterals. Particle counts were obtained from an electronic particle counter (HIAC, High Accuracy Instruments Division). After combining data from all the samples (157 samples of 19 aqueous drug products, 10 units per each sample), the upper 95% confidence limits of the means at 25 μm and at 10 μm were those listed above.

At that time, of the more than 500 official SVI products in the USP, 134 of these products (65) meet the following criteria for selection of products subject to particulate matter limit:

1. The drug is usually administered via the artery or vein or intrathecally.
2. The drug is likely to be used continuously or repeatedly for a course of treatment.
3. Drugs solely for emergency use, for diagnostic procedures, for anticancer therapy, or for episodic use are excluded.

While many laboratories preferred to employ the LVI microscopic method for counting particles in SVI products, the USP XXI introduced the use of an electronic liquid-borne particle counter system. Initial controversy over the test resulted in a postponement of the test becoming official until July 1985. The major complaint of the new USP method centered around the use of the HIAC-Royco electronic particle counter. Like any electronic counting device, the HIAC cannot identify and characterize particles, cannot accurately measure a particle's longest dimension (i.e., it measures all particles as spheres), will count silicon and air bubbles as particles, and standardization/calibration of the HIAC can be difficult. Also, many manufacturers objected to being forced to use an instrument that at that time was available from only one major U.S. supplier.

Other concerns over the proposed USP test for SVI particulate matter included lack of a sufficient database from which limits were established; lack of validation of the USP proposed method; the basis for requiring particle limits for some products but not for others in individual monographs; problems with specific details in the calibration, preparation, and determination sections of the test; and the lack of consistency between the LVI and SVI tests for particulate matter. These are discussed in more detail. The USP (Section 788) requirement for particulate matter in SVIs became effective in 1986. The test called for the use of an electronic liquid-borne particle counter system utilizing a light obscuration–based sensor

with a suitable sample-feeding device. The USP recommended Pacific Scientific, makers of HIAC/Royco particle counters, and using sensors manufactured by Russell Laboratories.

ELECTRONIC PARTICLE COUNTERS

The limitations of human inspection and microscopic analytical methods in the detection of particulate matter in injectable products have necessitated the use and advancement of electronic particle-counting methods in the pharmaceutical industry. In 1986, the USP adopted for the first time an electronic particle-counting method to be used in particulate matter testing of small-volume injections. Much controversy over the type, standardization, and limitations of electronic particle-counting methods has continued over the years. The 1987 International Conference on Particle Detection, Metrology, and Control concluded with the general perception that there remained many measurement problems with electronic particle counters (66). However, the 1990 Particle Conference closed on a strongly optimistic note that the basic error mechanisms have been identified, and that accurate, replicable particle data are within reach (67). These continuing advancements and problems will be reviewed in this section.

Two major advantages of electronic particle counters are their automated characteristics and the rapidity at which they accomplish particulate measurement. Two major disadvantages hinder electronic particle analysis from becoming a more acceptable means of measuring particulate contamination: They cannot differentiate among various types of particles, and they measure particle size differently from microscopic methods. These advantages and disadvantages are described in greater detail after first looking at the basic principles of different types of electronic particle-counting methods.

Principle of Electrical Resistivity (Coulter Counter)

The Coulter Counter (68–70) (Fig. 3.7) detects particles by measuring the change in electrical resistance produced when

Fig. 3.7 Photograph of Coulter Counter model ZM. (Courtesy Coulter Electronics, Inc., Hialeh, Florida.)

a particle displaces a part of the electrolyte solution residing between two electrodes. The change in resistance is directly proportional to the volume of the particle (70). The Coulter Counter therefore treats a particle as a three-dimensional object. This can be contrasted to the light-blockage principle, which views a particle in two dimensions and thus calculates area rather than volume of a particle.

Coulter Counters employ an aperture tube with a known micrometer opening that is immersed in a volume of parenteral solution. The ratio of particle diameter to orifice diameter should be less than 0.3 for the direct proportionality of resistance change and particle volume to be valid (71). For example, a 50-μm or greater aperture tube should be used for counting particles in the 10-μm size range.

Particle analysis must take place in a controlled clean

room under high-efficiency particulate air (HEPA)–filtered air to minimize environmental particles entering the sample solution. The aperture tube is immersed in the intravenous solution, which must be electrolytic. If not, an electrolyte solution (e.g., sterile sodium chloride for injection) must be added. This presents a major disadvantage in the use of the Coulter Counter if an electrolytic solution must be added. The solution itself may add a significant number of particles to the sample solution. Appropriate blank controls must be utilized to subtract the particulate contribution caused by the added electrolytic solution.

The instrument employs a manometer to sample from 500 μl to .2 ml of the intravenous solution. Counts measured in this extremely small volume may be too low (10–20 particles) for useful statistical accuracy of sampling (72). Air bubbles adversely affect accurate counting. Air bubbles are avoided by either minimized agitation during sampling or application of a vacuum before measurement. Electrical background noise also contributes to some error in actual counting of submicronic particles.

Sample solution is pulled through the aperture of the Coulter Counter solution tube and flows between two electrodes. The change in resistance, proportional to particle volume, creates a signal that is relayed to a threshold analyzer. The threshold analyzer has been previously calibrated so that only pulses of voltage exceeding the threshold position are counted. The pulses generated are displayed on an oscilloscope by electronic amplification. Voltage pulse heights are proportional to the amplifier gain and aperture current of the instrument and the resistance changes due to the passage of the particles (24).

The signal produced is proportional to the volume of electrolyte solution displayed by the particle. The count display is a function of volume directly or the diameter of a sphere of equal volume (73). The Coulter Counter can count up to 5000 particles per second using the Coulter principle of one-by-one

counting and sizing. Size distributions can be accurarely determined over a range of 0.5 to 800 μm, depending on the proper selection of optimal glassware.

Particulate matter in the subvisible size range present in intravenous solutions can be detected easily and rapidly by the Coulter Counter (71,74–78). Because of its electrical resistivity principle, the Coulter Counter especially applies in the determination of particulate contamination in parenteral electrolyte solutions such as those containing sodium chloride. Coulter Counters obtain particle size data with no indication regarding the shape or composition of the particles. The diameter of particles measured by the Coulter Counter is a mean spherical diameter. Since particles found in intravenous solutions are usually not spherical, it is important for the orifice dimension of the Coulter aperture tube to be much greater than the size of particles monitored by the counter. Acicular particles having lengths much smaller than the diameter of the aperture orifice will produce more accurate pulse heights having magnitudes closely corresponding to the total volume of the particle.

Principle of Light Obscuration (HIAC)

A schematic representation of the light obscuration principle is shown in Fig. 3.8. A tungsten lamp produces a constant collimated beam of light that passes through a small rectangular passageway and impinges onto a photodiode. In a clear passageway, the light intensity received by the photodiode remains constant. Liquids can flow through the passageway between the light source and the photodiode. If a single particle transverses the light beam, there results a reduction in the normal amount of light received by the photodiode. This reduction of light and the measurable decrease in the output from the photodiode is proportional to the area of the particle interrupting the light flow. Thus, the light-obscuration principle measures particle size based on the diameter of a circle having an equivalent area.

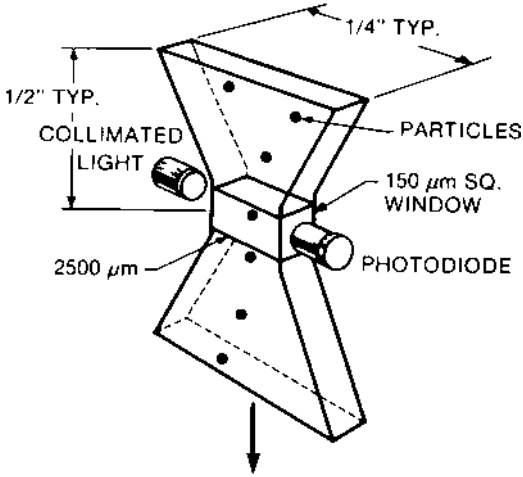

Fig. 3.8 Schematic representation of the light obstruction principle. (Courtesy HIAC/Royco, Menlo Park, California.)

HIAC particle counters employ the light-blockage principle in the detection and quantitation of particulate matter in parenteral solutions (see Fig. 3.9). These instruments count approximately 4000 particles per second. HIAC counters used sensors having size measurement ratios of 1:60. In other words, 1-through-60 micrometer sensor can measure particles from 1 to 60 μm, while a 2.5-through-150 micrometer sensor can measure particles ranging from 2.5 to 1.50 μm. Channel numbers on the counter are selected and calibrated according to the size range desired.

Increasingly, over the past several years, HIAC systems have progressed in technological advances and user application in the particle analysis field. Advances for using HIAC particle counters have outweighed the disadvantages. Lantz et al. (79) were among the first to publish results of HIAC analyses of parenteral solution particulate contamination. In conclusion, the advantages and disadvantages of using the HIAC particle counter are given below.

Fig. 3.9 HIAC/Royco's System 8003 for parenteral particle counting to comply with the requirements of microscopic particles. (Courtesy HIAC/Royco Division of Pacific Scientific Company, Silver Springs, Maryland.)

Advantages

1. Particles are counted automatically.
2. Parenteral solutions, either electrolytes or nonelectrolytes, could be counted.
3. The instrument was easy to calibrate and use.
4. Replication of counts was good.
5. The volume of samples could be varied as desired for counting.
6. Dilution method of counting permitted counting of both "clean" and heavily contaminated solution.
7. Direct method of counting permitted counting of crystallized soluble particles.

Disadvantages

1. Instrument is relatively expensive compared to equipment used for counting by optical microscope.
2. Particulate contaminants cannot be identified.
3. Large and/or fibrous particles may block the sensor opening.
4. Air bubbles are counted as particulate matter.
5. Dilution method of counting does not permit counting of crystallized soluble materials because dilution solubilizes crystals.

Principle of Light Scattering

When a beam of light strikes a solid object, three events occur: Some of the light is absorbed, some of the light is transmitted, and the rest of the light is scattered. Scattered light is a composite of diffracted, refracted, and reflected light. Particle counters that operate on the basis of light scattering are designed to measure the intensity of light scattered at fixed angles to the direction of the light beam. A schematic example is given in Fig. 3.10.

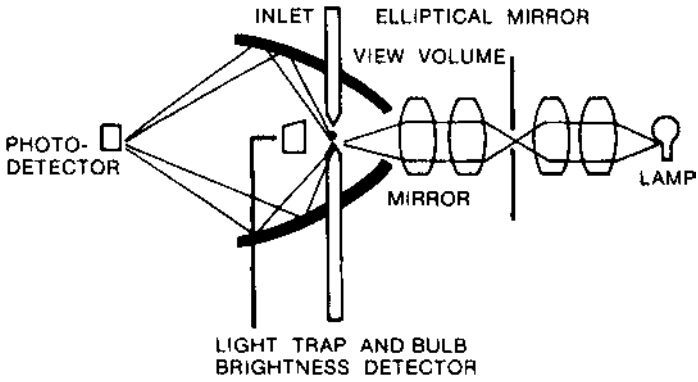

Fig. 3.10 Schematic representation of the light-scattering principle. (Courtesy Climet Instruments Co., Redlands, California.)

Table 3.8 Commercially Available Particulate Measurement Systems for Parenteral Use

Type of system	Model evaluated by the FDA[a]	Mechanism of measurement	Comments
Climet Instruments, P.O. Box 1760, Redlands, CA 92372	Model CI-1000	Light obscuration	Excellent large-particle detection
Coulter Electronics, 13960 NW 60 Street, Miami Lakes, FL 33014	Model ZM/P	Resistance modulation	Not recommended by the FDA for parenterals Large errors in measuring flakes and fibers
HIAC/ROYCO, Pacific Scientific, 2431 Linden Lane, Silver Springs, MD 20910	Model 4103	Light obscuration	Past recommendations in USP <788>

Kratel Instruments, D7250, Leonburg, Stuttgart, Germany	Boblinger Strasse 23	Light obscuration	Good large-particle detection
Met-One, 481 California Avenue, Grants Pass, OR 97526	Model 214	Forward light scattering, laser based	Laser diode light source
Particle Measuring Systems, 1855 S. 57th Court, Boulder, CO 80301	IMOLV/SOPS 100	Forward light scattering	Laser diode light source
Russel Laboratories, 3314 Rubio Crest Dr., Altadena, CA 91001	RLV 1-50H	Detector only	FDA recommended detector with HIAC

Source: Ref. 81.

[a] G. S. Oxborrow, A comparison of particle counters from five manufacturers, presented at the May 1987 Meeting on Liquid Borne Particle Inspection and Metrology, Washington, DC.

As a liquid flows into a light-sensing zone, particles in the fluid scatter light in all directions. The scattered light is directed onto a system of elliptical mirrors that then focus the light onto a photodetector. The light trap seen in Fig. 3.10 is designed to absorb most of the main light beam photons.

Met One and Climet particle counters represent examples of counters operating under this principle. Met One particle counters are laser-based particle counters that have become very popular instruments in the pharmaceutical industry both for airborne and liquid-borne particles. For liquid samples, particles in the liquid deflect bursts of laser light energy to a solid-state photodiode in which each burst of light is converted to a pulse of electrical energy. The electrical pulses are proportional in height to the particle size. Advantages of laser light particle counts include sample rates of 100 ml per minute, 3500 counts per milliliter, and simultaneous measurement of six particle sizes.

Davies and Smart (80) reported on rapid assessment of particle levels in small-volume ampule products with good reproducibility using the scattered-light-based particle counter. Advances and disadvantages of counters based on light scattering are similar to those identified for the HIAC counter described above. Table 3.8 provides a summary of commercially available electronic particulate measurement systems.

LIGHT OBSCURATION PARTICLE COUNT TEST

All SVI products containing 100 ml or less are required to pass the USP test. This includes reconstituted solutions. Products exempted from the USP test are injections intended solely for intramuscular and subcutaneous administration or products for which the label specifies that the product is to be used with a final filter.

Prior to using the USP procedure, three preliminary tests are to be done:

1. Determination of sensor resolution—Use monosize 10-μm particle standards to ensure that particle size distribution (manual method) or voltage output distribution (electronic

method) does not exceed the standard particle size by more than 10%.
2. Sensor flow rate—Certify that the actual flow rate is within the manufacturer's specification for the particular sensor used.
3. Sample volume accuracy—Since particle count varies directly with the volume of fluid sampled, sampling accuracy must be known and be within ± 5%.

The USP procedure provides specific directions and requirements for sample preparation, environmental conditions for conducting the testing, various necessary equipment to be used, glassware and closure cleaning, particulate control test, calibration of the particle-counting instrument, and determination of particulate matter in the product.

Test Environment

At a minimum, the light obscuration apparatus must be located within the confines of a HEPA-filtered laminar flow workbench. However, most industrial particle test laboratories have separate and contained work areas where these instruments are located. Furthermore, strict standard operating procedures are followed for entering the work area, with personnel appropriately gowned and samples introduced following procedures to remove all extraneous particulate matter.

All glassware, rubber closures, and other samples entering the testing environment must be scrupulously cleaned as described in the USP. Final rinsing of all materials is performed using filtered distilled water with the filter porosity 1.2 μm or finer.

Prior to determining particle counts of the actual samples, blank counts are determined using a clean vessel of the type and volume of the sample. Fifty ml of filtered distilled water are placed in the vessel; the vessel is agitated, degassed by sonification, allowed to stand, swirled by hand, and then three consecutive samples of at least 5 ml are withdrawn and counted. The first sample count is discarded, but the second two sample counts are used to determine acceptability of the

environment. USP requires that there are no more than 10 particles of 10 μm or larger and no more than 2 particles 25 μm or larger in the combined 10-ml sample.

Test Procedure

Prior to placing test samples within the confines of a laminar flow work area containing the light obscuration particle counter, the exterior surfaces of the samples are rinsed with filtered distilled or deionized water. Since samples need to be protected from environmental contamination after cleaning until analyzed, having a specially built clean room or module offers significant advantages. Technique to withdraw samples is very important to minimize particle contamination and introduction of air bubbles. Rubber closed vials may be sampled either by needles penetrating the closure or by completely removing the closure. This also holds true for vials containing sterile powders in that diluent can be added either via needle penetration or first removing the rubber closure. If samples are pooled, then it is better to remove the rubber closure.

If the volume of the product in the container is less than 25 ml, then at least 10 units of product are used for the LO test. Each unit of product is inverted at least 20 times and shaken vigorously to suspend the particles properly. The 10 units are opened, and the contents are pooled into a cleaned container. The total volume in the pooled container must be at least 20 ml. The pooled solution is degassed by sonification for at least 30 seconds, then left standing so that the air bubbles dissipate. The pooled container is then gently swirled and three aliquots of at least 5 ml of solution are withdrawn and injected into the LO counter sensor. The first sample measured by the electronic counter is not counted, but the next remaining samples are.

If the volume of the product in the container is 25 ml or more, the same procedure is followed as above to mix, suspend, and degas the solutions. Here, only three units of product are sampled, with each sample at least 5 ml. The first sample into the counter is discarded.

Freeze dried and other sterile powdered filled samples are reconstituted by first removing the rubber closure without contamination, using filtered water or other appropriate filtered diluent at the required volume. The rubber closure is then replaced, and the product is manually agitated to ensure complete dissolution of the drug product. Reconstituted samples can be pooled at described above, with the total volume of pooled sample being at least 20 ml. Invert the pooled product container 20 times prior to withdrawing sample; again, the first portion into the counter is discarded.

Calculations

USP provides calculations for pooled and individual samples from small-volume injections and calculations for individual unit samples from large-volume injections. The formulas are

Small-volume pooled samples:	PV_t/V_an
Small-volume individual samples:	PV/V_a
Large-volume individual samples:	P/V

where P is the average particle count obtained from the portions analyzed, V_t is the volume (ml) of pooled sample, V_a is the volume of each portion analyzed, V is the volume of the tested unit, and n is the number of containers pooled.

Interpretation

The injection meets the requirements of the USP subvisible particle test if the average number of particles present in the units tested does not exceed the following limits (see Table 3.15 for worldwide compendial requirements)

	≥10 μm	≥25 μm
Small-volume injections (particles per container)	6000	600
Large-volume injections (particles per ml)	25	3

If these limits are exceeded, then USP requires that the product must be tested by the microscopic particle count test.

MICROSCOPIC PARTICLE COUNT TEST

The present USP method provides both qualitative and quantitative data on particulate content in LVI solutions. Particles not less than 10 μm can be counted, sized, and described in terms of their shape and, at times, their nature (for example, a cotton fiber, piece of glass, or metal sliver). Photographs of the filter membrane further provide a permanent record of the particulate test results.

Considerable care and skill are required for preparing the membrane, cleaning the glassware and equipment used in the procedure, and using the microscope. This presents a major disadvantage and motivates pharmaceutical manufacturers to develop and validate alternative methods employing automation, electronic counting instrumentation, or both.

Procedure

Laminar Air Flow Hood

All operations and manipulations must be performed under a certified laminar air flow (LAF) hood equipped with HEPA-filtered air in a Class 100 environment. Laminar flow hood certification was discussed in Chapter 1.

Working in a laminar air flow environment can never replace the necessity for rigid clean technique in sample preparation and analysis. Prior to conducting a test, the hood must be cleaned with an appropriate solvent, preferably 70% ethanol or 70% isopropyl alcohol. The HEPA filter itself is not cleaned because of potential damage to the filter surface.

The hood should have a built-in sink or some accommodation for collection and disposal of solvent used in the filtration process.

Introduction and Use of Equipment in the Laminar Air Flow Hood

The USP demands the use of "scrupulously" clean glassware and equipment for the particle test. The word *scrupulous* means the following:

1. Rinse glassware and equipment successively with (a) warm detergent solution, (b) hot water, (c) water, and (d) isopropyl alcohol. The first supplement of the 19th edition of the USP listed a fifth rinse with trichlorofluoroethane (Freon 113). Freon was eliminated in the 20th edition procedure because of concern about its toxicity in a closed environment and harm to the ozone layer.
2. Rinsing technique is important. Glassware and equipment must be rinsed starting at the top of the vertically held object and working downward in a back-and-forth manner. Water rinsing may be done outside the LAF hood, but the final isopropyl alcohol rinse must be performed within the hood.
3. After rinsing, all objects must dry under the hood upstream of all other operations. This helps to ensure that few, if any, extraneous particles adhere to the drying object.

Rubber Gloves

The USP requires the use of suitable, nonpowdered gloves for the particle test. Gloves are important in protecting the hands from the dehydrating effects of isopropyl alcohol. However, gloves may create more problems than they solve. Using gloves of improper size will promote problems in careful handling of glassware and equipment. Gloves also produce a false sense of security, resulting in less than ideally careful manipulations in the LAF hood. The greatest potential limitation of gloves is the contribution they can make to particulate contamination, even after adequate rinsing. Thus, this requirement continues to be controversial.

Membrane Filter and Assembly

Membranes

The USP specifies the use of a gridded or nongridded, black or dark gray filter or a filter of suitable material compatible with the product. The filter must have a porosity of at least 1.0 μm.

Explicit instructions are provided in the USP for rinsing the membrane filter. In the 19th edition of the USP, Freon was used as the rinsing agent. In the 20th edition, water replaced Freon. Rinsing of a vertically held filter (using forceps) is accomplished using filtered water sprayed from a pressurized container. Rinsing of the membrane with filtered water starts at the top of the nongridded side, sweeping a stream of water back and forth across the membrane surface from top to bottom. This process is repeated on the gridded side of the membrane. Pressure exceeding 2 psi may damage the delicate membrane.

The rinsing solvent is checked for particle counts, serving as the blank determination in the testing portion of the USP procedure. It must be assumed that no dispensing vessel will provide a particle-free solvent. While the membrane filter on the nozzle will effectively remove particles above the rated porosity of the filter (usually 1.2 μm), particles on the downstream side of the filter on the nozzle will shed into the dispensed solvent. Of course, there is always the possibility of a misplaced or torn membrane filter on the dispenser nozzle.

Filter Assembly

The appropriately rinsed membrane filter is placed with the grid side up on the filter holder base. Great care is taken when the filtering funnel is situated on the base so that the membrane is not rumpled or torn. Prior to placing this assembly on the filtering flask, the unit is rinsed thoroughly and carefully with filtered water from the pressurized solvent dispenser. After allowing time for the rinse fluid to drain the filter, the apparatus is then secured on top of the filter flask.

Test Preparation

Containers to be tested for particulate matter must be inverted 20 times before the contents are sampled. Agitation has been shown to affect particle size distribution (82), so the 20-fold inversion procedure must be consistent. After rinsing the outer surface of the container with filtered water, the closure

is removed. One can never be certain that removal of the closure will not introduce extraneous particles. Careful aseptic and clean technique must be adhered to as much as possible.

After the closure has been carefully removed, the contents are swirled before 25 ml are transferred to the filtering funnel. After standing for 1 minute, a vacuum is applied to filter the 25-ml sample. An additional 25-ml sample of water is then applied to the sides of the funnel to rinse the walls of the funnel. The stream of filtered water should not hit the filter membrane for fear of tearing the membrane. The rinse fluid then is filtered via vacuum. Unfortunately, particles tend to adhere to the underside of the filter assembly top and to the O rings used between the filter base and filter funnel.

The funnel section of the assembly is carefully removed. The membrane is lifted away from the base using forceps and placed on a plastic Petri slide containing a small amount of stopcock grease or double-sided adhesive tape. The cover of the Petri slide is placed slightly ajar atop the slide to facilitate the membrane drying process. The slide then is placed on the micrometer stage of the microscope for visual analysis.

Particle Count Determination

Examination of the entire membrane filter surface for particulates may be accomplished using a precisely aligned and calibrated microscope. The microscope should be binocular, fitted with a 10× objective, and have one ocular equipped with a micrometer able to measure accurately particles of 10μm and 25 μm linear dimension. Incident light should be set at an angle of 10° to 20°, although an angle of 30° and 35° has been reported to be more effective in illuminating the membrane surface inside a plastic Petri slide (83). Calibration of microscope micrometers based on a National Bureau of Standards primary standard-stage micrometer has been described by Lanier et al. (84).

Particles are counted under 100× magnification with the incident light at an angle of 10° to 20°. Obviously, this is a

slow and tedious process requiring patience and dedication on the part of the microscopist. Use of higher magnification, up to 400×, may be necessary occasionally to discern discrete particles from agglomerates or amorphous masses (83). Sometimes, particles not visible with dark field reflected light are very easily observed by means of bright field illuminization at 45° polarization.

Two sizes of particles are counted, those having effective linear dimensions 10 μm or more and 25 μm or more. The counts obtained from the sample membranes are compared to counts obtained from a membrane treated exactly like the sample membrane minus the filtration of the product sample. Blank membrane counts rarely are zero. However, if 5 or more particles 25 μm or larger and/or more than 20 particles 10 μm or larger are counted on the blank membrane, the test is invalidated, and it signifies a serious problem in one or more of the following areas: poor technique, filter breakdown in the solvent dispenser, poorly cleaned membranes, poorly cleaned filter assemblies, and/or HEPA filter leaks. The problem must be resolved before particle testing can resume. If the USP limit of not more than 12 particles per milliliter 10 μm or larger and not more than 2 particles per milliliter 25 μm or larger is exceeded, the large-volume injection product fails the USP test for particulate matter. For small-volume parenterals, the test fails if more than 3000 particles/container are 10 μm or larger and/or 300 particles per container are 25 μm or larger.

Analysis by microscopic techniques suffers from several disadvantages: it is very time-consuming, requires technical expertise, and, because of the manpower requirements, can be very expensive, The major method for determining subvisible particulate matter in parenteral solutions, including reconstituted sterile powders, is the light obscuration technique. However, if any dispute arises regarding fulfillment of USP particulate matter specifications, such disputes must be settled by applying the official USP microscopic method.

LVI particulate matter standards in other countries gov-

erned by other compendia are reviewed in a section at the end of this chapter.

AUTOMATED MICROSCOPIC PARTICULATE MATTER TEST

Human variables unavoidably decrease the accuracy, precision, and reproducibility of manually measuring particulate matter using the USP microscopic method. As pointed out by Clements and Swenson (85), considerable time and concentration are required even under ideal circumstances for the microscopist to perform the necessary operations to obtain a clear and accurate view of a particle's longest dimension. Viewing multiple particles adds to the complexity of time and concentration. Parallax errors (differences in sizing a particle when seen from two different points not on a straight line with the particle) decrease accuracy, especially when measuring smaller (e.g., 5–10-μm) particles. In summary, the success of application of the manual microscopic technique is directly proportional to the microscopist's efficiency, which in turn is dependent on his or her speed, concentration, and alertness.

To minimize or eliminate the human factor in the USP particulate matter test, a number of electronic particle-counting instruments have been developed, refined, and computerized for rapid and relatively accurate and reproducible particle measurements. However, the main disadvantage of electronic particle counting from a regulatory point of view is a lack of adherence to the official USP test. As previously noted, electronic instrumentation for particle counting is permitted to satisfy USP particle test requirements for large-volume injectables. However, in cases of controversy, the USP microscopic method must be the final judge.

The nearest equivalent automated system to the USP manual microscopic method is a system called image analysis. The system described here differs from the automated inspection systems described in the section, "Visual Inspection: Automatic Methods." Image analysis is not a 100% final con-

tainer inspection system. Rather, this system introduces automation after the large-volume parenteral sample has been filtered and the membrane prepared. Particle analysis of the filter membrane is performed by a computer-controlled microscope and television system.

The Quantimet automated image analysis system has been fully explained in the literature (85). According to the USP procedure, the membrane filter, following filtration, is mounted in a plastic filter holder and placed securely on the microscope stage plate. An external fiber-optic illuminator provides low-angle, high-intensity illumination with directional control to satisfy USP requirements and to create optimum particle contrast against the filter background. Optically interfaced to the microscope is a high-resolution television camera. Each field of the filter surface imaged by the microscope is scanned by the camera, which produces a digital picture containing geometric and densitometric information. The camera signal is processed by the central processor, in which data representing the longest dimension of each particle on the filter surface are fed to the output computer for processing and presentation. Results can be displayed on the video monitor printed to provide a permanent hardcopy record, and stored on magnetic disks for future recall. To meet USP requirements, data generally are reported as the number of particles having effective linear dimensions equal to or greater than 10 μm and equal to or greater than 25 μm.

Millipore Corporation developed a particle measurement computer system similar in theory, instrumentation, and application to the Quantimet system (86). The πMC system consists of (a) a microscope and television camera that illuminate and observe the sample on the filter membrane, (b) a computer module that receives the video signals from the television camera and applies the appropriate logic to count and measure particles in the viewing area, and (c) a viewing monitor that subsequently receives the video signal, reconstructs the field of view, and prints the desired particle data at the top of the monitoring screen.

The advantages of automated microscopic analytical systems are

1. They conform to the USP procedure for particle analysis of large-volume injectable solutions.
2. Particles are counted, sized, and shape-characterized with much greater speed and precision compared with the manual microscopic method.
3. Efficiency and reproducibility are increased, while tedium is eliminated.
4. Permanent records in the form of particle data and photomicrographs can be obtained.
5. Operation of these systems requires minimal technical and manipulative skills.

Barber et al. have published a number of interesting articles attempting to improve methodology for conducting particulate matter evaluation in parenteral solutions. They have criticized and suggested improvements in the USP particulate test (87) and have suggested new methods, such as automated microscopy (88).

Developments in video-camera technology and image-processing software have made possible the development of automatic systems for measuring particle size, counting particles in various size ranges, and even classifying particles according to shape or elemental content (if the imaging system uses an electron microscope with elemental detection (89).

Photon-correlation spectroscopy is a laser-based technique that detects scattered laser light from a sample and analyzes individual photon pulses with an autocorrelator. The random motion of individual particles with respect to one another produces intensity variations because of interference effects of the laser light. These variations are measured as diffusion coefficients, and from these values, particle sizes can be calculated if a shape is assumed. Particle counts cannot be calculated, but particle sizes between 1 nm and 1 μm can be determined without destroying the sample. Also, measurements are absolute; no calibration is necessary.

Halographic imaging is another laser-based technique in which halographic images of particles (5 μm and larger) in solutions can be measured. This technique also permitted three-dimensional shape-mapping of particles and holds considerable promise for nondestructive particle detection.

Sedimentation field-flow fractionation employs a centrifugal field for the separation of particles of different sizes. The operation of this instrument is similar to that for chromatography. The result is a high-effficiency separation of a particle mixture according to weight. Depending on the experimental conditions, field-flow fractionation can separate particles in the 0.01–1 μm or 1–100 μm ranges.

COMPARISON OF MICROSCOPIC AND ELECTRONIC PARTICLE-COUNTING METHODS

The comparisons discussed in this section include methods capable of quantitating particulate contamination, that is, microscopic and electronic methods. Comparisons involving visual inspection, both manual and automated methods, were discussed in the section "Comparison to Other Particle Inspection Methods."

Difficulties in comparing particle-counting methods result from differences in the way in which different methods determine particle size and distribution. For example, the microscopic method measures size as the longest linear dimension of the particle. The principle of light blockage, utilized by the HIAC particle counter, expresses size as the diameter of a circle of equivalent area as the actual area consumed by the particle. Particle counting by electrical resistance (Coulter Counter) treats the particle as a three-dimensional object and measures the volume consumed by the particle. Thus, the microscope, HIAC, and Coulter Counter methods size particles in one, two, and three dimensions, respectively.

An excellent theoretical discussion by Schroeder and DeLuca (73) showed that it is virtually impossible to correlate instrumental and microscopic particle counts directly for ir-

Table 3.9 Summary of Sphericity Correction Factors Based on Longest Linear Dimension

Shape	D_O longest dimension	D_H horizontal projection	D_A light blockade	D_V electrolyte displacement
Sphere	1.00	1.00	1.00	1.00
Cube (1:1:1)	1.00	0.90	0.95	0.88
Equant (3:2:1)	1.00	0.88	0.81	0.62
Prolate ellipsoid (2:7:1)	1.00	0.87	0.61	0.52
Flake (4:4:1)	1.00	0.90	0.81	0.55
Rod (3:1 diameter)	1.00	0.81	0.62	0.52
Fiber (rigid, 10:1)	1.00	0.64	0.36	0.25

Source: Ref. 73.

regularly shaped particles. As seen in Table 3.9, as long as the particle is a sphere, all methods will size the sphere equally. However, as the particle shape deviates from sphericity, the size measurement by the three alternate approaches will differ, sometimes drastically, from the value obtained by the USP microscopic method. For example, if the solution sample contained 50 ellipsoid particles with their longest linear dimension equaling 10 μm, the HIAC will yield a count of $50 \times 0.61 = 30.5$ particles. In fact, this HIAC value may be an overestimate because the 0.61 correction factor considers only size (10 μm), not the actual number of particles. Assuming the size-count relationship follows the conventional log-log relationship, the theoretical HIAC count of 50 ellipsoid 10-μm particles would be only 14.4 particles. Figure 3.10 provides the explanation. The USP microscopic method follows a log-log distribution, yielding a straight-line slope between 10 μm and 25 μm for its pass/fail criteria of 50 particles/milliliter at 10 μm and 5 particles/milliliter at 25 μm. Assuming the HIAC method follows the same log-log distribution between 10 μm and 25 μm, its slope will be parallel to the USP slope. However, the HIAC correlation factor for ellipsoid particles theoretically

is 0.61 that of the USP method. Thus, the starting point for the HIAC method is not 10 μm, but 6.1 μm at the 50-count position of the log-log graph. Therefore, following a parallel relationship with the slope of the USP method, the HIAC method yields a theoretical particle count value of 14.4 particles at the point intersecting the vertical line from the particle size of 10 μm.

This same logic can be assumed for the Coulter Counter method. From Table 3.10 and Fig. 3.11, the size correction factor of the Coulter Counter for ellipsoid particles is 0.52. Applying the same log-log relationship for a 50 particle/milliliter sample, the Coulter Counter will yield a count of about 9.7 particles 10 μm or larger.

Hopkins and Young (90) were the first investigators to publish actual particle size and number data from typical parenteral solutions analyzed by the microscope, HIAC, and Coulter Counter methods. Some of their results are reproduced in Tables 3.11 and 3.12. Table 3.11 shows that the Coulter Counter yielded particulate counts that deviated between +9.2% and +40.8% from counts obtained with the mi-

Table 3.10 Count Comparison Between USP and Electrolyte Displacement Methods

			Displacement count at	
Shape	USP count	SCF	10 μm	6.6 μm
Sphere	5	1.00	5.00	14.20
Cube	5	0.88	3.63	10.30
Equant	15	0.62	4.51	12.82
Ellipsoid	10	0.52	1.93	5.49
Flake	5	0.55	1.11	3.16
Rod	5	0.52	0.97	2.75
Fiber	5	0.25	0.15	0.44
Total/milliliter	50		17.30	49.16

Source: Ref. 73.

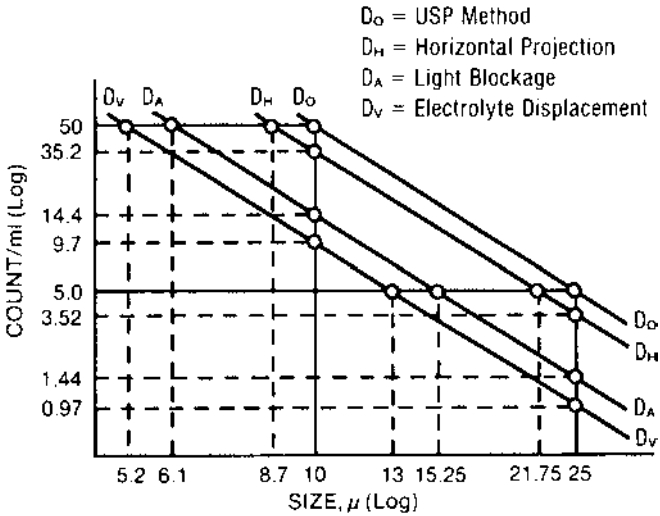

Fig. 3.11 Log count versus log size corrections for sizing and counting prolate ellipsoids. (From Ref. 73.)

croscope. The HIAC counts were between −19.2% and +3.8% from those of the microscope. Table 3.12 demonstrates that (a) the agreement among the three methods was acceptable, especially considering their different mechanisms of particle sizing and the fact that these data all fell well within the Australian particle standard (100 particles/milliliter = 5 μm)

Table 3.11 Comparison of Microscope, Coulter, and HIAC Data, Showing Total Counts of Particles Larger than 5 μm

Sample	Microscope	Coulter	HIAC
5606A, Supplier A	6285	6867	5080
5606A, Supplier B	19,364	23,534	18,000
5606A, Supplier B	5113	7200	5020
5606A, Supplier A	4285	4635	3660
5606A, Supplier A	6675	8715	6930

Source: Ref. 90. Data from Technical Documentary Report ML-TDR-64-72, Air Force Materials Laboratory, Wright-Patterson Air Force Base, Ohio.

Table 3.12 Particle per Milliliter of Isotonic Saline Solutions in the Range of 5–50 μm

		HIAC	Coulter	Microscope
MFG A S-1[a]	Average	10.8	17.4	11.9
MFG B S-2[a]	Average	19.2	15.1	11.4
MFG B S-3	Average	7.4	9.7	8.6
MFG B S-4		9.9	6.6	7.9
		10.3	9.2	8.1
		8.7	7.4	8.3
		9.6	7.7	8.1
Filled water blank	Average	0.9		

Source: Ref. 90.
[a] Air bubbles in solution.

present at that time (USP standard was not official at that time), and (b) great error is produced in both Coulter and HIAC assays when no attempt has been made to exclude air bubbles from the sample solutions. These instruments do count air bubbles as particles; thus, vacuum techniques must be applied to eliminate air bubbles before any instrumental particle counting is performed.

A similar conclusion was reached by Rebagay et al. (91) in that an automatic particle counter can be used in place of either a polarizing microscope or an image-analyzing system for routine particulate matter monitoring of various particle systems (AC Test Dust, polystyrene spheres, antibiotic, electrolyte and large-volume parenteral solutions). However, to do this, the particle counter must be carefully calibrated with particles that possess morphological and optical characteristics similar to the particles of interest. An example of their data for the measurement of particle content of various intravenous infusion solutions is given in Table 3.13.

Lim et al. (92) filtered various small-volume parenteral solutions and counted particles using the manual counting method under the microscope and the electronic Millipore method. In products with relatively few particles, both meth-

Table 3.13 Particulate Matter Determination of Some Intravenous Solutions by Automatic and Microscopic Methods

Infusion solutions	Microscopic method[a]		Image analyzer[b]	Automatic[c]	
	I	II		I	II
5% Dextrose	2869 ± 336	2604 ± 180	2936 ± 275	2673 ± 192	1748 ± 172
5% Dextrose + 0.2% NaCl	2003 ± 127	1928 ± 222	2058 ± 159	1813 ± 125	1223 ± 80
5% Dextrose + 0.45% NaCl	1863 ± 67	1708 ± 119	1642 ± 102	1680 ± 89	879 ± 23
Lactated Ringer's solution	2078 ± 304	2009 ± 200	2096 ± 190	2039 ± 156	1032 ± 105
0.9% Sodium chloride	1247 ± 136	1201 ± 99	1205 ± 271	1250 ± 201	705 ± 176
10% Protein hydrolysate	7374 ± 267	7408 ± 231	4509 ± 160	7185 ± 879	4252 ± 507

Source: Ref. 91.

[a] Reichert Zetopan Universal Microscope. I, Incident polarized light and polycarbonate as substrate; II, incident bright-field lighting and cleared white cellulosic substrate.

[b] TIMC computer measurement method with cleared white cellulosic substrates.

[c] HIAC counter, calibrated with (I) AC Fine Test Dust and (II) polystyrene-divinylbenzene spheres.

ods gave similar results. In products containing a high number of particles in the size range of 5–25 μm, the electronic method detected more particles. These authors concluded that the electronic method was preferable because of its greater rapidity and precision. A somewhat similar conclusion was made by Blanchard et al. (93) in comparing the microscope and the Prototron laser beam (using the light-scattering principle). With solutions containing abundant particles of the small-size range, the particle counter gave more reliable and accurate results than did the microscope.

CURRENT ISSUES WITH ELECTRONIC PARTICLE COUNTERS

Knapp and DeLuca (81) listed problems encountered to some degree with all available instrumentation used to measure particulate matter (Table 3.14). Many of these problems were also discussed by Knapp (94), who also proposed action steps to overcome these problems. Today, many of these problems have been resolved with advances in the instrumentation available.

Table 3.14 Problems Encountered with Electronic Particle Counting Systems

Low sample volume handling capacity—imposes sampling errors due to loss of particles during sampling manipulation before and during analysis
Shape-dependent signals
Inadequate particle size range
Specification of inappropriate measurement limits—number of particles counted too low for measurement accuracy or the concentration of particles is too high for sensor capability
Inability to distinguish between particles, microbubbles, and insoluble microdroplets (e.g., silicone oil)
Flow problems in sensing zone, resulting in random orientation of particles

Source: Ref. 81.

Barber (95) published an excellent paper detailing the limitations of LO particle counters as required by the USP for measuring particulate contamination in SVIs. The single greatest obstacle in using LO counters is their inaccurate measurement of both particle number and particle size. This is not because of design flaws or engineering defects with these counters, but rather because of the basic principle on which these instruments operate. As discussed in the section "Principle of Light Obscuration," particle counts result from a series of interactions between a particle moving at high velocity and an intense light beam in the counter's sensor. Whenever a particle crosses the light beam, the intensity of light that reaches the photodiode is reduced, and an amplified voltage pulse is produced. The amplitude of the pulse is approximately proportional to the area of the particle projected onto a plane normal to the light beam, and the particle size is recorded by the counter as the diameter of a sphere having an equivalent projected area. When particles are few, large (>5 μm), and spherical, good numerical accuracy is possible. However, when particles are many, small (<5 μm), and nonspherical, inaccuracies will result. A particle's residence time in the view-volume usually is too short to allow the sensor to detect more than one aspect of the particle; consequently, the LO measurement is based on the light that is obscured by the particle according to its orientation when it enters the counter's view-volume.

Solution flow rates greatly affect count accuracy. Slower rates result in longer pulse durations, increased probability of electronic noise effects on count pulse and possible increases in apparent particle size. Faster flow rate pulses may not rise to full height, resulting in undersizing (95).

Nonspherically shaped particles produce significant errors in the sizing accuracy of electronic particle counters. Because particles of irregular shape are viewed in random aspect as they pass through the sensor of a counter, the size recorded typically will be less than that defined by the maximum area of light obscuration. Such an effect is shown in Table 3.9.

As differences between the refractive index of the particle

and the refractive index of the solution containing the particle increase, the measured particle size will increase. A particle in water will have a greater refractive index, between the two than the same particle in a concentrated solution of dextrose. Thus, these particles in water will be measured by the light obscuration sensor to have greater size and greater number than the same particles in the concentrated dextrose solution.

Calibration errors can occur because calibration is done with monosize spherical latex particles, which provide a very narrow range of known monoshape particle size. This introduces a calibration bias when measuring actual and largely unknown sizes and shapes of particles in parenteral solutions. The error introduced nearly always results in particle measurements being smaller than they should be. However, to attempt to calibrate counters with nonspherical particles adds greater difficulties because of their nonuniformity, dispersal difficulties, and differences in chemical composition and optical properties; the calibration "value" would be practically meaningless.

Coincidence effects occur when two or more particles are counted as a single larger particle. This problem can be most easily detected by comparing dilutions of the same sample; if an increase in total counts occurs with the diluted sample, coincidence counts are probably the cause. Eradication of coincidence effects is difficult; the only reasonable method for obtaining valid data with such solutions is to do microscopic analyses.

Immiscible fluids and air bubbles counted as particles are other sources of error for light obscuration and other electronic particle-counting methods. The primary source of immiscible fluid is silicone, usually very small (< 1 μm) microdroplets. Only in significant numbers do silicon microdroplets produce significant errors in particle measurement. Air bubbles are also problematic, but the USP provides for a method of "degassing" the sample using ultrasonification. Such degassing does not remove all microscopic bubbles or reduce the dissolved air content in the solution.

Sampling variability, as with any quality control test re-

lying on sampling procedures, must also be recognized as a source of error with electronic particle counting. Sampling-associated factors that adversely affect particle counting are caused by particle stratification effects, by a small sample volume relative to the total sample volume, and by the low numbers of particles per milliliter that typically are counted in a parenteral solution. Adequate agitation of the product container prior to collecting samples must be properly done to minimize the effects of sampling variability.

In August 1990, the USP invited the Particulate Matter Committee of the PMA QC Section to meet and discuss various suggestions for improving the USP test for particulate matter (Section 788) (96). The USP had a three-stage proposal: (a) an increase in the minimum volume of sample to be tested, (b) an increase in the minimum number of test units to be tested, and (c) use of composite versus individual testing. The committee decided that none of these changes needed to be made. However, the committee did agree to do three new assignments: (a) provide data and information regarding the possibility of tightening the light obscuration limits in the USP, (b) seek and provide silicone assays that could be used for testing at Stage 2, and (c) study the adoption and the Improved Microscopic Assay for Stage 3.

FACTORS AFFECTING ACCURATE PARTICLE TESTING

Nearly every scientific paper featuring the use of a particle test method, be it visual, microscopic, electronic, manual, or automatic, has to alert the reader to one or more major limitations to the method. These limitations have been addressed in this chapter. For example, visual examination by human beings is limited by its tedium and subjectiveness. Microscopes often are improperly calibrated. Electronic particle counters count air bubbles as particles. For LVIs, the USP relies on membrane filtration, by which particles from the equipment, environment, or personnel involved in conducting the test inadvertently become deposited on the filter.

Other problems exist that can potentially cause inaccu-

rate particle test results regardless of the test used. Ernerot (97) pointed out that the particle contents of injection containers vary considerably between the date of production and a later date when the same containers are tested again. It was found that storage causes particle agglomeration. Mechanical agitation breaks up the agglomerates, resulting in counts that cannot reproduce the original count or replicate one another on the same date of testing. Freshly prepared solutions seemed to give more stable counts. It was suggested that only the manufacturer, who can reproduce the handling of its products, use particle counting as a meaningful control method.

Agitation or shaking will increase the number of particles in a parenteral solution. Blanchard et al. (82) found that the slope and number of particles per milliliter greater than 1 μm in a log-log plot of number against diameter depended on the degree of agitation. Agitation of LVI by 20 hand inversions, as required by the USP procedure, removed particulate matter from the surface of the container, thus increasing the total number of particles greater than 1 μm. Yet, the relative size distribution of particles was not altered significantly. Agitation for 30 minutes disintegrated agglomerates, greatly increased the number of particles with diameters less than 1 μm, and brought about a corresponding decrease in the number of particles exceeding 1 μm in diameter. Particle-counting procedures must be carried out that do not impose a sheer force on the particles and affect the reproducibility of the test results.

Temperature affects the number of particles found in parenteral solutions. As shown in Fig. 3.12 (98), particle number increased as a function of temperature and time. Interestingly and without clear explanation, a decrease in the particle number occurred after 120 hours of storage at all temperatures. Since the particle size range studied was 2.33 to 5.02 μm (using the Coulter Counter), it is possible that particle agglomeration occurred, resulting in a decrease in particle number at these smaller diameters, but an increase in particle counts at

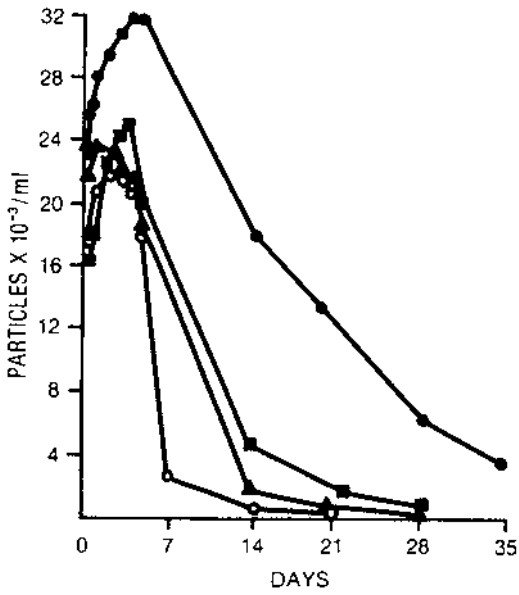

Fig. 3.12 Number of particles between 2.33 and 5.02 μm found in resting plastic bags after shaking for 30 hours as a function of time and temperature. Key: ■, 35°C; ○, 45°C; ▲, 55°C; ●, room temperature. (From Ref. 98.)

larger sizes. These same investigators found that glass containers produced fewer particles than plastic containers under similar storage and handling conditions.

INTERNATIONAL COMPENDIA STANDARDS FOR PARTICULATE MATTER CONTENT IN PARENTERAL SOLUTIONS

Table 3.15 is based on an article by Lotteau (99) and a review of the available compendia updated to reflect requirements as of 2002. In comparing the requirements from different compendia, the following should be noted:

1. The EP now has particulate matter requirements for small-volume injectables that were effective in 2000.

Table 3.15 Comparison of Compendia for Particulate Matter Standards

Compendia	LVI/SVI	Method	Limits*
USP	LVI	Microscopic	12 parts/ml ≥ 10 μm 2 parts/ml ≥ 25 μm
	SVI	Light obscuration	6000 parts/container ≥ 10 μm 600 parts/container ≥ 25 μm
BP	LVI	Coulter Counter	1000 parts/ml ≥ 2 μm
European	Solutions ≥ 100 ml Test A	Light obscuration	25 parts/ml ≥ 10 μm 3 parts/ml ≥ 25 μm
	Solutions ≤ 100 ml Test B	Light obscuration	6000 parts/container ≥ 10 μm 600 parts/container ≥ 25 μm
Japan	LVI	Microscopic	20 parts/container ≥ 10 μm 2 parts/ml ≥ 25 μm
	SVI	Light obscuration	1000 parts/container ≥ 10 μm (proposed)

* parts = particles.

2. The EP differentiates particulate matter requirements in small-volume injectables whether the injectable product is a ready-to-use solution or a powder that has been reconstituted into a solution.
3. Only the BP designates the use of the Coulter Counter as the electronic method for measuring particulate matter in large-volume solutions.
4. Each compendia has slightly different acceptability standards with respect to either particle size and/or particle number.

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4

Package Integrity Testing

INTRODUCTION

The assurance of patient safety requires that all parenteral product packaging prevent the ingress of microbial contaminants. While this is certainly critical, it can be said from a broader perspective that package integrity is the measure of a package's ability to keep the product *in* and to keep potential contaminants *out*. The "product" necessitating containment is typically thought of as either the formulated liquid or the solid drug product inside the package. But the product may also include the gas headspace environment within the package. Such is the case for products requiring the maintenance of a rarefied gas headspace or a partial pressure environment to ensure either the product's stability or the package's functionality. And while parenteral packaging must prevent potential microbial contamination, it must also prevent the ingress of unwanted environmental debris, chemicals, or particulates.

Package integrity is a requirement to be met throughout the product's life cycle. The verification of a package's ability

to maintain adequate integrity should begin in the development stages of a product. Package integrity assurance should continue after product approval by manufacturing within the acceptable ranges of established critical specifications and process controls. Manufactured product integrity may be supported by the additional use of appropriate tests conducted in conjunction with production operations. Shelf life package integrity tests of stability samples provide final evidence of marketed product quality.

Regulations passed primarily by the U.S. Food and Drug Administration (FDA) have helped to drive the incorporation of appropriate package integrity tests as part of the development process and ultimate manufacture of parenteral products. A variety of tests is available for use by the pharmaceutical industry to measure parenteral product package integrity. While all testing approaches have some value, each also has drawbacks. Test methods vary in sensitivity and capability. This is complicated by the fact that products differ in package integrity requirements. Even different seals within the same product/package system may have different integrity demands. For instance, a package system that includes a parenteral delivery device may consist of a closure to contain the product and to prevent liquid microbial ingress, as well as an attachment for product administration only intended to prevent airborne microbial contamination at the time of use. Finally, the stage of the product's life cycle may stipulate different types of testing to be performed; package validation studies may require more exhaustive and sensitive testing than routine manufacturing. Therefore, no single test can be recommended for all parenteral package integrity testing.

Fortunately, research and development of package integrity tests has escalated in recent years. By gleaning a general understanding of leakage concepts and by utilizing published integrity test data, it is possible to more logically design, assemble, and validate integral parenteral product packaging.

REGULATIONS AND GUIDANCES

U.S. Food and Drug Administration

Prior to the mid-1990s only sterility of the packaged product was required by the FDA as verification of package integrity. We have seen a dramatic change in the FDA's requirements for package integrity verification over the last several years, as several final and draft industry guidance documents issued by the FDA include discussions of package integrity. The first of these documents was issued in 1994; "Guidance for the Submission Documentation for Sterilization Process Validation in Applications for Human and Veterinary Drug Products" (1). This landmark guidance required that the microbial barrier properties of a parenteral product package be verified and stated that sterility testing alone is insufficient for this purpose. This guidance included the following requirements relating to package integrity:

1. Validated integrity studies must be performed, and data must be included in the submission.
2. Experiments must stimulate the stresses anticipated during product life, including maximum sterilization cycle(s).
3. Physical, chemical tests are allowed as well as the more traditional microbial challenge studies.
4. Each sterile package seal barrier should be separately evaluated and validated.
5. The sensitivity of the method used should be specified and provided.
6. Microbial integrity should be demonstrated over the shelf life of the product.

This was followed by a Draft Guidance in 1998: "Container and Closure Integrity Testing in Lieu of Sterility Testing as a Component of the Stability Protocol for Sterile Products" (2). This document solely addressed the need for incorporation of appropriate, adequately validated container closure integrity tests, beyond sterility testing, to verify a

package's microbial barrier properties as a part of a sterile product's stability protocol. Both microbial challenge methods as well as physicochemical test methods were permitted. Stability studies included those for pending new product applications, investigative or unlicensed products, and approved/licensed products. *Products* included biologicals, human and veterinary drugs, and medical devices. Tests were recommended annually and at product expiry; sterility was still required for product release and at product expiry. This draft was never issued as a final guidance, and very likely it will not be as package integrity is addressed in later guidance documents that focus on specific product development stages and regulatory document submissions. However, it did much to clarify the agency's thinking on package integrity.

Again in 1998, the FDA issued a Draft Guidance for Industry: "Stability Testing of Drug Substances and Drug Products" (3). This draft, still in active status at the time of this chapter's preparation, covers stability studies needed throughout the pharmaceutical product's life, including Phase 1, 2, and 3 development and new drug application (NDA) and abbreviated new drug application (ANDA) submissions. Approved product stability protocols and stability testing to support postapproval changes are included as well. Container integrity is addressed in Section VII (Specific Stability Topics, C. Microbiological Control and Quality). Here, it is stated that "appropriately sensitive, adequately validated" integrity tests are to be performed annually and at expiry throughout the product's stability program to demonstrate a package's ability to prevent microbial ingress.

The FDA issued another Draft Guidance for Industry in February 1999: "INDs for Phase 2 and 3 Studies of Drugs, Including Specified Therapeutic Biotechnology-Derived Product, Chemistry Manufacturing, and Controls Content and Format" (4). This Draft Guidance recommends the inclusion of container closure microbial integrity tests in Phase 3 stability studies to demonstrate package integrity during drug product shelf life. Along with the submission of the test to the FDA,

a rationale for test selection relating to container integrity should be provided.

A final FDA Guidance for Industry was issued in May 1999: "Container Closure Systems for Packaging Human Drugs and Biologics" (5). This guidance document covers the requirements that must be met for container closure systems intended for packaging all human drug and biological products. A demonstration of container closure integrity is one portion of the information required to justify a package's suitability for its intended use. Parenteral packages must demonstrate integrity against microbial and environmental contamination. But in addition, packages for some products must prevent solvent loss, exposure to reactive gases, or absorption of water vapor. The integrity tests used and the data generated are to be submitted as part of the chemistry, manufacturing, and controls section of a new drug application.

In conclusion, it is clear that the FDA understands that package integrity is a critical requirement for all pharmaceutical products. As a matter of consumer safety, industry is being lead by the FDA to verify the ability of parenteral product packaging to prevent microbial contamination for products in later stages of clinical development as well as those approved for marketing. While microbial challenge methods have traditionally been used for this purpose, the FDA will permit the utilization of other approaches with the submission of supportive scientific rationale. In addition, integrity verification beyond microbial barrier testing is required during development for products that require the minimization of solvent loss, exposure to reactive gases, or absorption of water vapor.

European Union Regulatory Bodies

European regulatory requirements say little to date about container closure integrity of parenteral or sterile pharmaceutical products. Regulations provide for package integrity verification of parenteral vials to be supported by the performance of

sterility tests as part of the stability program. More specific information is described in the EU 1998 "Rules Governing Medical Products in the European Union, Pharmaceutical Legislation" (6). These GMP regulations require that the sealing or closure process be validated. Packages sealed by fusion (e.g., ampules) should be 100% integrity tested. Other packages should be sampled and checked appropriately. Packages sealed under vacuum should be checked for the presence of vacuum.

While not as detailed as the FDA Guidances, it is evident that the EU Rules also require the verification of parenteral product package seal integrity. It is important to note that the EU Rules specifically require 100% product testing for fusion-sealed packages, sampling and testing of all other packages, and vacuum verification for packages sealed under partial pressure. The FDA Guidances, on the other hand, do not mandate the extent of testing within a production lot or call for the verification of vacuum presence.

PDA Technical Report No. 27

Due to the complexity of pharmaceutical product/package systems and the confusion that can arise when trying to select the most appropriate integrity tests, an effort was made by the Parenteral Drug Association (PDA) to publish a technical resource to offer clarification. The end result was Technical Report No. 27, "Pharmaceutical Package Integrity," prepared by a task force of scientists from both industry and academia (7). This report begins with a summary of package leakage concepts and critical leak specifications. It discusses the need to consider package integrity for the life of the product, beginning in early product development. Eighteen different integrity tests are described and thoroughly referenced. These are linked to a decision tree to help the reader in selecting the most appropriate methods. While neither an official regulatory requirement nor guidance document, PDA Technical

Report No. 27 is included in this section as it can serve as a valuable reference when selecting and developing package integrity tests.

LEAKAGE

Definition

Leakage occurs when a discontinuity exists in the wall of a package that can allow the passage of gas under the action of a pressure or concentration differential existing across the wall. Leakage differs from *permeation*, which is the flow of matter through the barrier itself.

Permeation is governed by Fick's laws of diffusion (Eqs. 1 and 2), for which permeation rate is a function of the permeant's concentration and its solubility in the barrier material as well as the molecule's physical ability to migrate through the barrier (8,9). For permeation to occur, the molecule must be adsorbed onto the barrier, then move through the material by dissociation and migration, and finally exit by desorption on the other side of the barrier.

Fick's first law assumes a barrier of infinitely small thickness, that is, a membrane:

$$J = -D(\delta C/\delta x)_t, \tag{1}$$

where

J = amount of diffusion, $g/m^2 \cdot s$
D = diffusion constant, m^2/s
C = concentration of diffusant, g/m^3
x = barrier thickness, m
t = time, s

In the case of a barrier of measurable thickness, the concentration gradient of diffusant varies across the thickness, and is continually changing with time, thus acting to change the flux. This situation is defined by Fick's second law (8,9), where

$$\delta C/\delta t = D(\delta^2 C/\delta x^2) \tag{2}$$

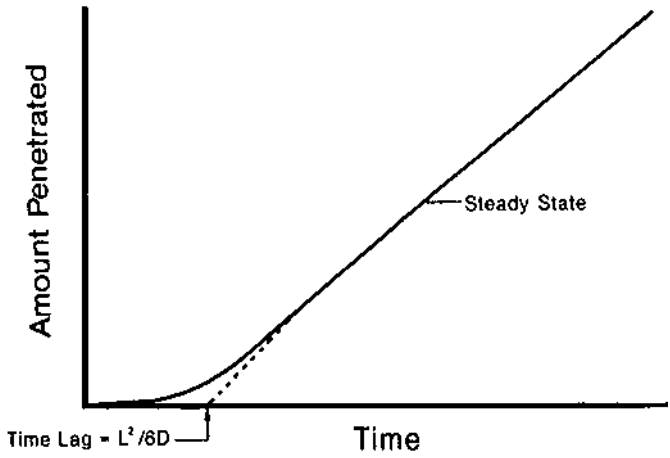


Fig. 4.1 Permeation flux versus time when the time lag for the penetration of the diffusant is a function of the barrier thickness L and the diffusion constant D. (Courtesy of the Parenteral Drug Association, Inc., Bethesda, MD.) (Source: Ref. 9.)

A graphic representation of permeation flux with respect to time is given in Fig. 4.1.

Leakage, on the other hand, is a mass flow phenomenon in which molecules move by convection and diffusion through a gap in the barrier. Solubility of the diffusant in the barrier material plays no part in either the convection or the diffusional flux associated with leakage. The driving force for convection of gases as well as liquids is the pressure differential across the pore or leak. The driving force for diffusional leakage is the concentration gradient existing across the leakage gap. This relationship is described in Eq. 3, where the total flux for species A is a function of the sum of the convective fluxes for all species and of the diffusion of A, which depends on the concentration gradient of only species A (10):

$$J_A = X_A(j_A + j_B) - (p)(D_A)(X_A)(1/x) \tag{3}$$

where

J_A = total leakage flux of species A, $g/m^2 \cdot s$
X_A = fractional amount of species A, dimensionless
j = convective flux of each species, A and B, $g/m^2 \cdot s$
p = density of mixture, g/m^3
D_A = diffusion coefficient of species A, m^2/s
x = barrier thickness, m

Diffusional Flux, Convective Flux, or Permeation?

Before trying to apply the theories of leakage flux and permeation to a particular package, it may be helpful to consider a few practical examples of how these phenomena relate to one another.

One example of leakage diffusional and convective flux acting simultaneously is a sealed parenteral vial containing a nitrogen headspace shipped in a partially pressurized cargo section of an airplane. On the ground under ambient conditions, oxygen will tend to diffuse into and out of the container through any leaks present, although more oxygen will diffuse into the container than out due to the partial pressure differential existing between the nitrogen vial headspace and the atmosphere. At 15,000 feet above sea level in the cargo section of a plane, the pressure outside the vial will be reduced, causing the differential pressure across the package seal to be about 6 psig. Under these conditions, oxygen will continue to diffuse into the vial; however, oxygen flow into the container will be restricted by the net positive pressure inside the vial, which will convectively force nitrogen and liquid product out.

Because convective flux is generally much greater than diffusional flux, leakage theory generally assumes a single-component system for which only convective flux occurs. In other words, only a total pressure gradient exists, and there is no concentration gradient of gas across the seal. One example of a parenteral package that may require the consideration of diffusional leakage is an inert gas flushed vial in which convective flux may approach zero, but enough diffusional leak-

age across the seal could result in the loss of headspace integrity. Another example is a wet-dry syringe, for which a rise in moisture level of the lyophilized powder could occur due to water vapor diffusional leakage moving from the "wet" chamber into the dry one. In addition, moisture trapped in the elastomer separating the chamber may permeate out of the elastomer, adding to the cake's total water content.

In conclusion, when considering the microbial barrier properties of a package, only leakage, not permeation, is a consideration. However, when considering the overall integrity of a parenteral package system, it may be necessary to consider the sum of leakage and permeation. For instance, if a product contains a volatile or solvent component that can sorb into the container, then permeation plus leakage needs to be considered. Or, if a parenteral package must maintain an inert gas or partial pressure headspace, both leakage and permeation may play a role in satisfactory package performance.

Leakage Units of Measure

Leakage is mathematically defined as the rate at which a unit of gas mass (or volume) flows into or out of the leak under specific conditions of temperature and pressure. For example, a carbonated beverage can may leak 10 cc of carbon dioxide in 3 months at 60 psig, or a submerged package may leak two bubbles per second of 1/8 inch diameter when pressurized to 40 psig.

The units of measure commonly used in many literature references to specify leakage rate are standard cubic centimeters per second (std cm^3/s or std cc/s). According to the international metric system of units (SI nomenclature), leakage is measured in pascal cubic meters per second ($Pa \cdot m^3/s$). In both expressions, units of gas mass (std cc and $Pa \cdot m^3$) indicate the quantity of gas (air) contained in a unit of volume at sea level atmospheric pressure (101 kPa). For very precise measurements, standard temperature of 20°C (293°K) is also specified. Unless temperature varies widely during an experiment,

Table 4.1 Leakage Units of Measure

$Pa \cdot m^3/s$[a]	Std cm^3/s[b, c]	Std L/day[c]	Air at 0°C kg/year
1	10	864	400
0.01	0.1	8.6	4
10^{-4}	10^{-3}	86×10^{-3}	4×10^{-2}
10^{-6}	10^{-5}	86×10^{-5}	4×10^{-4}
10^{-8}	10^{-7}	86×10^{-7}	4×10^{-6}

Other units:

1 microliter per second (μL/s) = 1.33×10^{-4} Pa · m^3/s

1 microcubic foot per hour (μft^3/hr) = 1.0×10^{-6} Pa · m^3/s

1 torr liter per second (torr L/s) = 0.133 Pa · m^3/s

[a] SI nomenclature.

[b] More commonly used nomenclature. Also abbreviated as std cc/s.

[c] Expressed as quantity of gas (air) in a unit of volume at sea level atmospheric pressure.

however, small changes due to temperature variation are insignificant compared to the potentially large differences in gas pressure. Today, std cc/s is the more common unit of measure, but because both units of measure are conventionally used, leakage rates given in this chapter are presented either in units of std cc/s or Pa · m^3/s, depending on the preference of the referenced resource. To convert to std cc/s from Pa · m^3/s, the SI units should be multiplied by a factor of 9.87 or approximately 10. These and other common leakage units of measure are summarized in Table 4.1 (11).

Leakage Modes and Flow Rates

There are three modes of convective flux leakage that describe the flow patterns demonstrated by leaking gas. Turbulent flow is very rapid leakage, followed by slower laminar flow (LF), and finally the even slower molecular or "Knudsen" flow. For capillary pores larger than about 10^{-4} cm in diameter, gas leakage is typically turbulent. Turbulent flow rates measure greater than 10^{-2} std cc/s (9,12). Laminar flow occurs for capil-

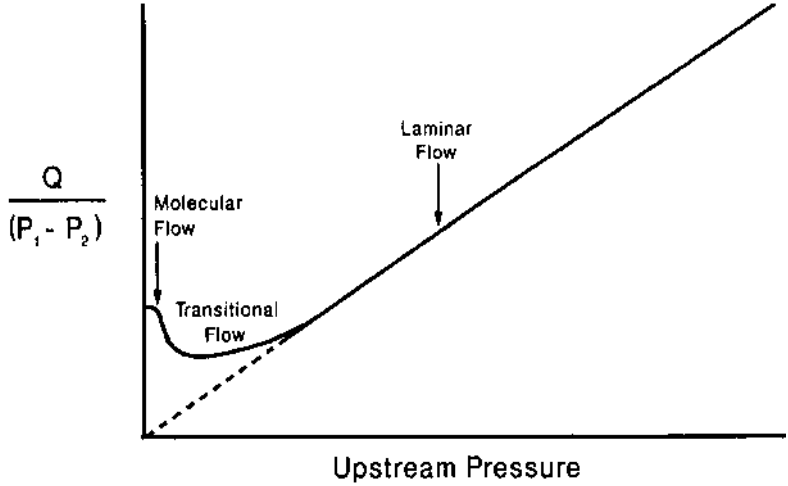


Fig. 4.2 Leak rate convective flux. (Courtesy of the Parenteral Drug Association, Inc., Bethesda, MD.) (Source: Ref. 9.)

laries about 10^{-4} cm in diameter, and measured leakage rates are approximately 10^{-3} to 10^{-6} std cc/s (9,10,12–14).

Molecular flow is most probable with leakage rates below 10^{-5} std cc/s (9,12). It is frequently seen in situations of very low gas pressure as well. Molecular flow leakage is so slow that it only describes leakage of gases and not liquids. The mathematical relationships between leakage flux and the differential pressure across the seal are described in Eqs. 4 through 6 and are illustrated in Fig. 4.2. More detailed equations can be found in the references cited above.

$$\text{Molecular: } Q = f(P_1 - P_2) \quad \text{most probably} < 10^{-5} \text{ std cc/s} \tag{4}$$

$$\text{Laminar:} \quad \text{most probably } 10^{-3} \text{ to } 10^{-6} \text{ std cc/s} \tag{5}$$

$$\text{Turbulent:} \quad \text{most probably} > 10^{-2} \text{ std cc/s} \tag{6}$$

where

Q = convective flux (mass/time)

P_1 = upstream pressure
P_2 = downstream pressure

"Leak Free"

When package integrity is critical for acceptable product performance, leakage is generally compared to a leak rate specification. For example, a pickup truck is "leak tight" if the truck bed keeps the smallest nugget of gravel on board. A pacemaker will "leak" during its implant life if it exhibits a leakage rate of greater than 10^{-9} std cc/s. Vacuum vessels meet a leak rate specification of no greater than 10^{-5} std cc/s (15).

Figure 4.3 illustrates approximate gas leakage rates and

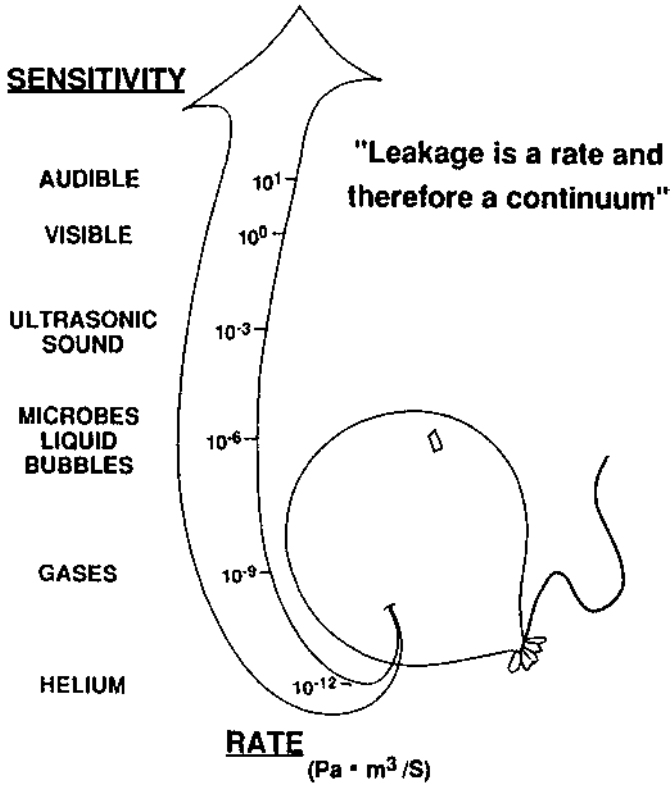


Fig. 4.3 Leak rate as a continuum.

their practical significance. This illustration points out that leakage is a rate and is therefore a continuum. The practical significance of a given leakage rate will depend on the nature of the substances contained in the package. Very rapid leakage is audible (i.e., the noise from a whistling teapot). As leaks get smaller, they become imperceptible to the human senses. As discussed in the next section, aqueous liquids have been shown to migrate through channel leaks of as small as about 10^{-8} Pa · m^3/s (10^{-7} std cc/s) measured by helium mass spectrometry. Most gases cannot pass through leaks smaller than about 10^{-9} Pa · m^3/s (10^{-8} std cc/s). Because helium gas can flow through the smallest holes; it is therefore useful as a tracer gas for detection of leaks as small as 10^{-12} Pa · m^3/s (10^{-11} std cc/s).

Expressions such as "leak free," "without leaks," or "leak tight" are meaningless when used alone. All package seals and closures leak to some degree. What is meant by those terms is that any leaks present are so small that they have no practical significance. In other words, leakage is occurring below the established leak rate specification.

ESTABLISHING LEAK RATE SPECIFICATIONS

Liquid-borne Microbial Leakage Specification

The leak rate specification for any given product/package system, also called the *critical leak rate*, is a function of the package's required protective and containment properties. For parenteral products, packages must minimally prevent the loss of product and prevent the ingress of microorganisms or external contaminants. Before establishing such a specification, the leak size that will permit product loss and microbial ingress must be known.

In the late 1980s, Morton et al. designed a series of tests to determine the gaseous, liquid, and microbial barrier properties of vial/closure compression seal systems (16,17). A simulated vial was sealed at various compression forces using elastomeric closures. The gaseous leakage rate was first measured

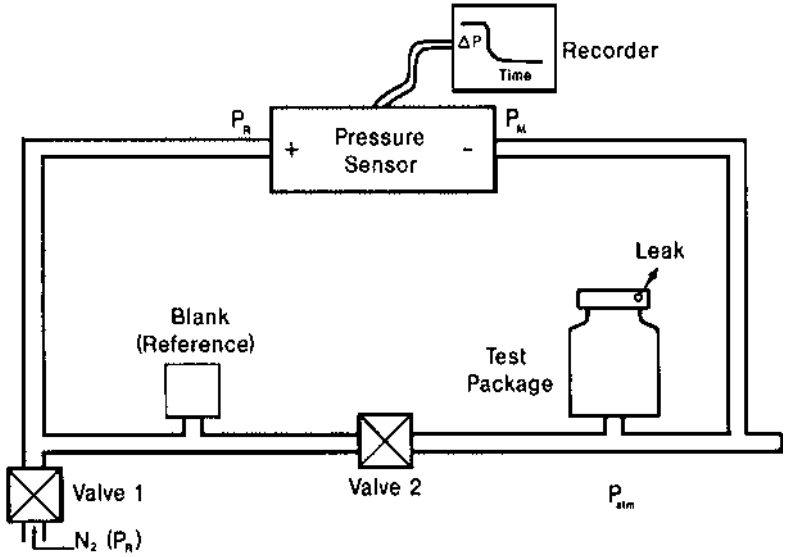


Fig. 4.4 Differential pressure laboratory test unit. Differential pressure decay measured between test vial side of manifold and reference side over time. (Courtesy of the Parenteral Drug Association, Inc., Bethesda, MD.) (Source: Ref. 16.)

across the seal using a laboratory-scale differential pressure testing apparatus (Fig. 4.4). With this device, various elastomeric closures could be applied to the test vial within a range of compression forces and tested for their ability to affect a seal. Leakage from the test package was measured by first opening both valves 1 and 2, flooding the manifold and test package with nitrogen to a target pressure of 3 psig, then closing both valves and measuring any detectable differential pressure change within the manifold over time. The leakage rates measurable with this system ranged from 10^{-3} to 10^{-7} Pa · m^3/s (or 10^{-2} to 10^{-6} std cc/s) at 3 psig differential pressure. Typical results obtained are illustrated in Fig. 4.5, in which leakage rates for one particular type of polymeric-coated elastomer are plotted against closure compression (17).

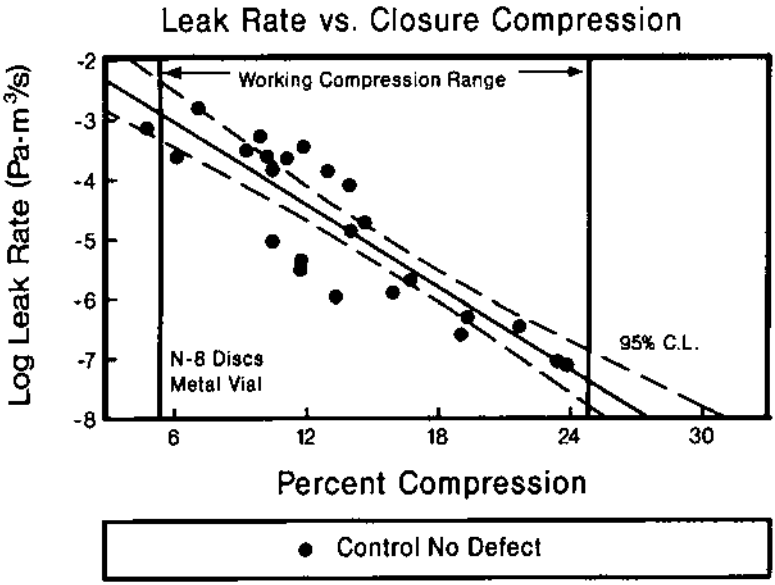


Fig. 4.5 Parenteral vial leakage rate versus percentage of compression of coated closures. Modified polypropylene (N-8) film-coated closures were used. (Courtesy of the Parenteral Drug Association, Inc., Bethesda, MD.) (Source: Ref. 16.)

After characterizing the leakage rates of model test packages, they were then transferred to a separate test manifold for evaluating the microbial barrier properties of the seal (Fig. 4.6). The test packages were filled with a saline lactose broth suspension of *Pseudomonas aeruginosa* at a minimum concentration of 3×10^8 colony-forming units per milliliter. The filled vials were inverted so that the finish area was immersed in sterile saline, then the vials were pressurized to 3 psig for 15 minutes to replicate the conditions of the differential pressure test and a liquid tracer test (see below). The immersion fluid was checked for the presence of the challenge microorganism by a filter plate count method. Figure 4.7a illustrates the incidence of microbial leakage for vials sealed at various closure compression levels superimposed on the gaseous leakage

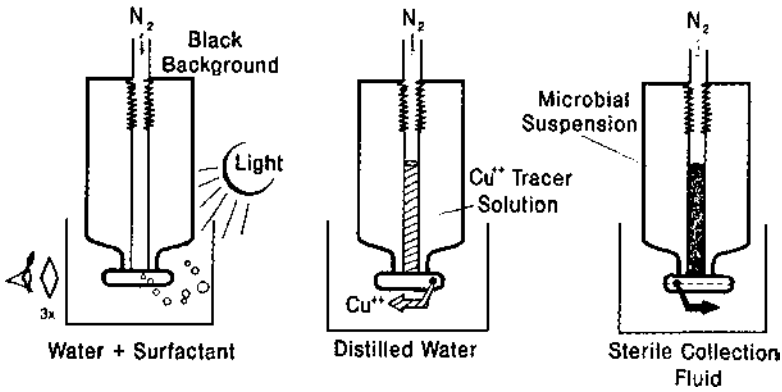
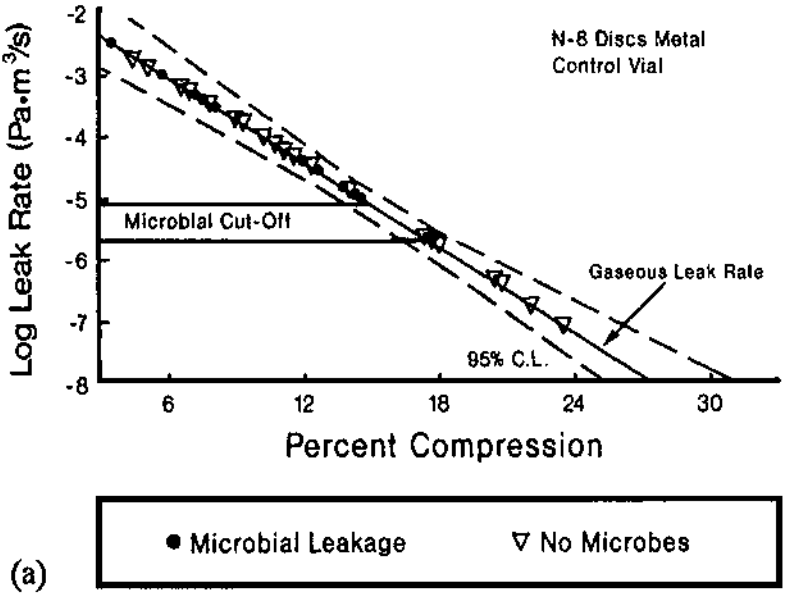


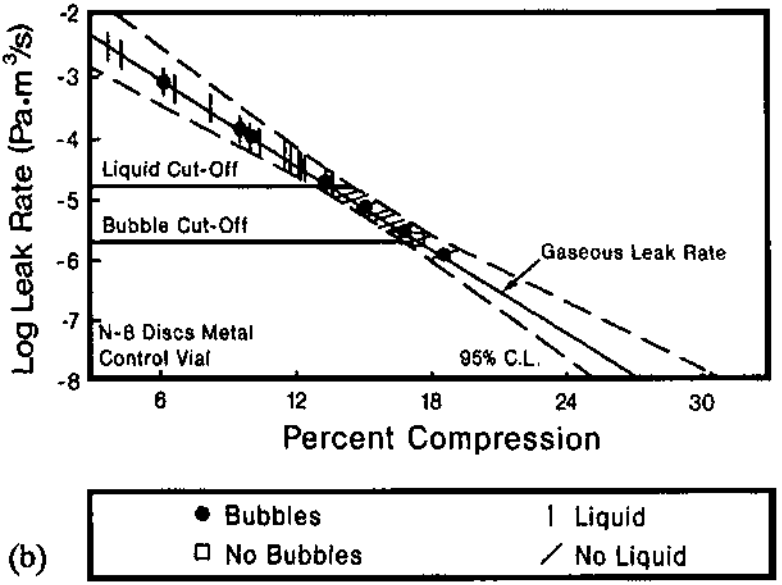
Fig. 4.6 Laboratory test units for bubble, liquid chemical tracer, and microbial egress leak test comparisons. (Courtesy of the Parenteral Drug Association, Inc., Bethesda, MD.) (Source: Ref. 17.)

rates measured by differential pressure decay. As illustrated, *P. aeruginosa* was incapable of passing through vial/closure compression seals that exhibited gas leakage rates of less than 10^{-5} Pa · m^3/s (or 10^{-4} std cc/s). Interestingly, there were vials that failed to allow microbial ingress even at gaseous leakage rates significantly higher than the critical leakage cutoff rate.

Later research by Kirsch et al. at the University of Iowa provided more exhaustive microbial challenge test results that correlated helium leakage rate to microbial ingress (18,19). While Morton et al. investigated leakage through a vial/closure compression seal, Kirsch and colleagues studied leakage through glass micropipettes of various sizes embedded in the walls of glass vials (Fig. 4.8). A population of vials containing holes ranging in size from 0.1 to 10 μm in diameter was flooded with helium and subsequently tested for helium leak rate using a mass spectrometry leak rate detector (18). The relationship demonstrated between helium leak rate and nominal leak diameter is shown in Fig. 4.9. These same vials were then filled with sterile media and immersion challenged for 24 hours at 35°C with a saline lactose suspension of 10^8 to 10^{10}



(a)



(b)

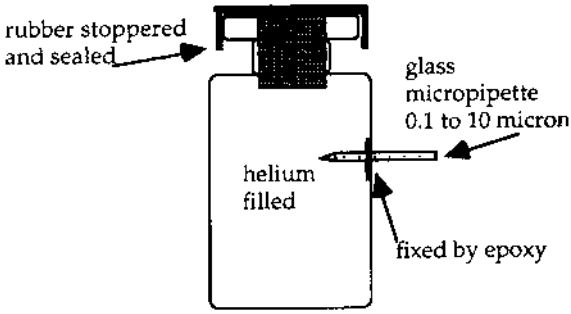


Fig. 4.8 Schematic description of the modified pharmaceutical vials used as test units for the evaluation of mass spectrometry–based helium leak rate measurements. (Courtesy of the Parenteral Drug Association, Inc., Bethesda, MD.) (Source: Ref. 18.)

colony-forming units of *P. diminuta* (*Brevundimonas diminuta*) and *Escherichia coli* (19). Prior to immersion challenge, the test vials were thermally treated to eliminate airlocks within the micropipet lumen and establish a liquid path between the microbial challenge media and the test units' contents. After immersion challenge, the test vials were incubated at 35°C for an additional 13 days.

The results showed that the probability of microbial ingress decreased as hole size and helium leakage rate decreased (Fig. 4.10). Even under such extreme challenge conditions, only 3 of 66 test vials with log leak rates less than −4.5 std cc/s failed the microbial challenge, which is consistent with the vial/closure interface leakage results reported by Morton et al. The probability of microbial ingress dramatically dropped from over 60% to about 10% within the helium log

Fig. 4.7 Parenteral vial comparative leakage test sensitivity results. (a) Microbial leakage versus differential pressure gas leakage; (b) liquid chemical tracer and bubble test leakage versus differential pressure gas leakage. (Courtesy of the Parenteral Drug Association, Inc., Bethesda, MD.) (Source: Ref. 17.)

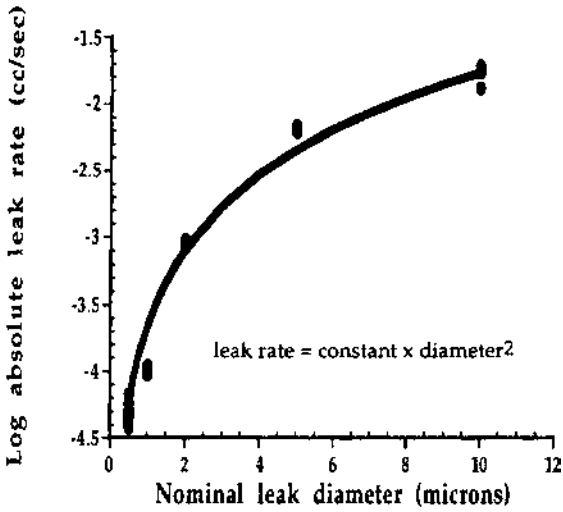


Fig. 4.9 The relationship between nominal leak size and the absolute leak rate. The absolute leak rate was determined using test units that contained a headspace composed only of helium. (Courtesy of the Parenteral Drug Association, Inc., Bethesda, MD.) (Source: Ref. 18.)

leak rate range of log -3.8 to -4.5 std cc/s, which roughly corresponds to a leak diameter range of 0.4 to 1.0 μm. Ingress no longer occurred in this study through holes with helium leakage rates between 10^{-5} and $10^{-5.8}$ std cc/s. Thus, the work by Kirsch and colleagues, backed by the results of Morton et al., is often cited to support a critical leak rate specification of anywhere from 10^{-5} to $10^{-5.8}$ std cc/s helium leak rate measured at 1 atmosphere differential pressure and standard temperature conditions for rigid, nonporous parenteral packages.

Liquid Leakage Specification

As stated, parenteral packages also need to prevent the loss of product or the ingress of external contaminates. Over two decades ago, a study was performed using metal cans in which microbial ingress was measured and correlated to water in-

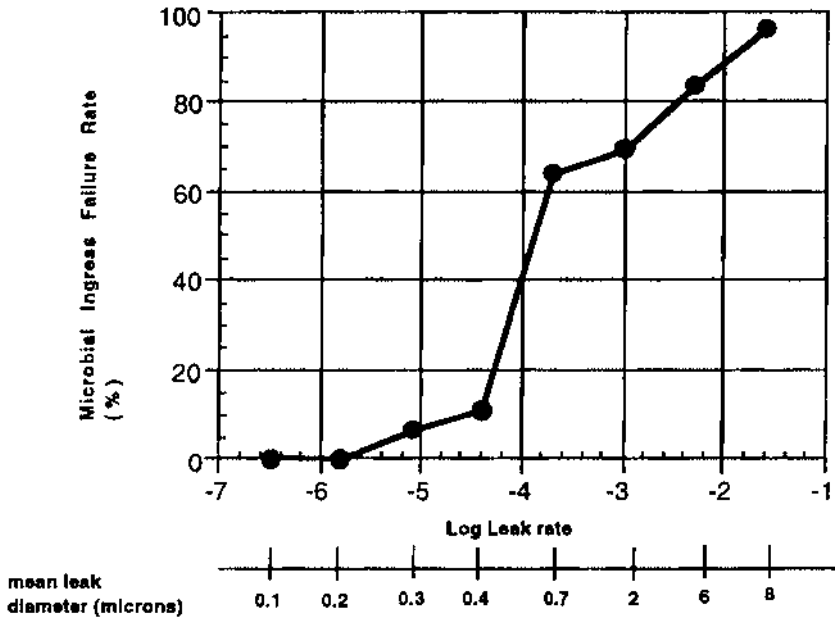


Fig. 4.10 The correlation of microbial failure rate (%) and the mean logarithm of the absolute leak rate and nominal leak diameter for modified small volume parenterals. The absolute leak rate (standard cubic centimeters per second) was determined by mass spectrometry–based helium leak rate detection. Microbial failure was measured by microbial ingress after 24-hour immersion in a bath (37°C) containing 10^8 to 10^{10} *P. diminuta* and *E. coli* organisms/milliliter and a 13-day, 35°C incubation. (Courtesy of the Parenteral Drug Association, Inc., Bethesda, MD.) (Source: Ref. 19.)

gress and helium gas leakage rate (20). Water ingress was determined using a manganese tracer added to the microbial challenge solution. In this case, water ingress was directly related to the ingress of microorganisms, which in turn was related to the log of the helium gas leakage rate measured through the cans.

This research sparked a similar study by Morton and colleagues, who investigated the ability of their model package's

vial/closure seal to prevent liquid leakage. The liquid challenge consisted of a copper sulfate tracer solution detectable by atomic absorption spectrometry assay (17). After filling the simulated vials with tracer solution, the finish areas of the vials were lowered into 10 ml of water, and a differential pressure of 3 psig was applied to accelerate potential leakage for a period of 15 minutes (Fig. 4.6). The test was calculated to be capable of detecting as little as 0.1 μl of copper solution in the water. The ability of this test to detect leakage was compared to that of a bubble test, the previously cited microbial challenge test, and differential pressure change leakage test. As seen in 4.7b, copper tracer solution was detected in all containers known to exhibit gaseous leakage rates greater than or equal to 10^{-5} Pa · m^3/s (10^{-4} std cc/s). This is consistent with older literature references, which state that aqueous leakage will not occur when dry air, at the same pressure, leaks at a rate as great at 10^{-5} Pa · m^3/s (21). Interestingly, liquid leakage detected with copper tracer was found to be a more reliable predictor of leakage than microbial challenge tests as copper was found in all vials with leaks above the critical gaseous rate, but not all vials above this leakage rate exhibited microbial leakage (contrasting graphs a and b of Fig. 4.7).

This research was again collaborated by Kirsch and colleagues using test vials with micropipets. As mentioned, prior to challenging the test vials with microbial suspension, the vials were thermally treated to ensure a liquid pathway through the micropipet. Adding magnesium tracer ion to the challenge solution and checking for its presence inside the test vials at the end of the incubation period by atomic absorption spectrometry verified the presence of liquid in the pipet leak path. This body of data provided an interesting correlation among helium leak rate, the probability of liquid leakage, and the chance of microbial ingress, as summarized in Ref. 22. In the test vial population, all vials demonstrating microbial ingress also contained magnesium ion tracer. However, there were a significant number of vials that allowed passage of magnesium tracer but did not allow microbial ingress. Logisti-

cal regression models were used to describe the probability of microbial or liquid tracer presence as a function of the logarithm of the helium leak rates (Fig. 4.11). The probability of liquid tracer ingress is greater than microbial ingress at leakage rates less than log −2 std cc/s, with liquid tracer detected for a few leaks as small as about log −7 std cc/s. The probability of liquid ingress decreased dramatically for leaks smaller than about log −5.4 std cc/s. These results support the contention that liquid penetration of a leak precedes and is a requirement for microbial ingress. Again, as with the research by Morton et al., microbial ingress is not a certainty even when a rather significant liquid pathway is present. It can be concluded that sufficiently sensitive liquid tracer tests can be a better predictor of a package's liquid and microbial barrier properties than microbial immersion challenge tests.

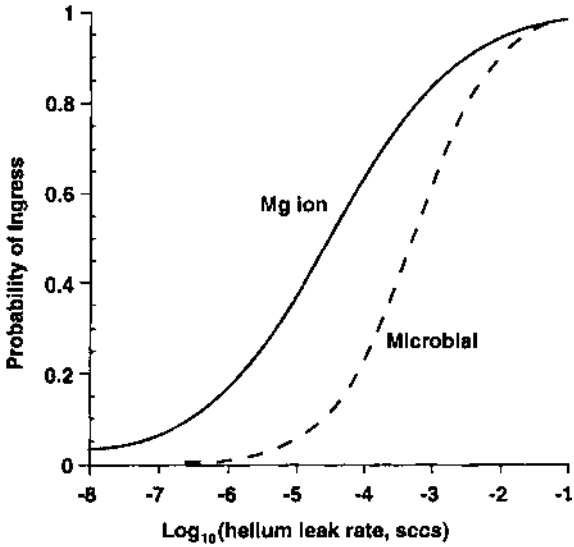


Fig. 4.11 Logistical regression models describing the probability of microbial or liquid tracer (magnesium ion) as a function of the logarithm of the helium leak rates. (Courtesy of the Parenteral Drug Association, Inc., Bethesda, MD.) (Source: Ref. 22.)

Gaseous Leakage Specification Limit

A container that must maintain a specified inert atmosphere content or vacuum level will most likely require a stricter leak rate specification. For example, suppose a parenteral vial with a headspace volume of 5 cm^3 is stoppered under an initial headspace vacuum level of 5 psia with a maximum allowable vacuum level of 10 psia at the end of a 2-year expiry.

If vacuum loss were to occur within this allowable range in an essentially linear fashion, the allowable leak rate could be simply calculated as

$$\text{Leak rate} = \text{Mass/Time or (Pressure} \times \text{Volume)/Time}$$
$$\text{Leak rate} = (10 \text{ psia} - 5 \text{ psia})(5 \text{ cm}^3)/2 \text{ years}$$

Converting to SI units,

$$5 \text{ psia} = 34{,}470 \text{ Pascals}$$
$$5 \text{ cm}^3 = 5 \times 10^{-6} \text{ m}^3$$
$$2 \text{ years} = 6.3 \times 10^{-7} \text{ s}$$

Therefore, the maximum allowable leak rate is

$$(3.45 \times 10^4 \text{ Pa})(5 \times 10^{-6} \text{ m}^3)/(6.3 \times 10^7 \text{ s}) \quad \text{or,}$$
$$2.7 \times 10^{-9} \text{ Pa} \cdot \text{m}^3/\text{s}$$

It should be noted, however, that if vacuum loss were allowed to proceed to atmospheric conditions, the leakage rate over time would be more sigmoidal than linear, with a tapering off of leakage rate as the vacuum in the vial approaches atmospheric conditions.

In the above example, the maximum acceptable leakage rate is defined according to SI units of $Pa \cdot m^3/s$. Alternatively, leakage can also be defined according to a product-specific end point. For example, a lyophilized product may contain an initial moisture level of 0.1% water, with a maximum allowable moisture level at expiry of 4.0%. If the mass of the lyophilized cake is 2.0 g, then the maximum allowable leak rate can be calculated as

$$\begin{aligned}\text{Leak rate} &= (\text{Mass of water pickup})/(\text{Expiry dating}) \\ &= (4.0\% - 0.1\%)\ (2.0\ \text{g})/2\ \text{years} \\ &= 39\ \text{mg of water per year}\end{aligned}$$

Again, this assumes that the maximum allowable limit for water content is not near the product's saturation point, and that moisture sorption by the lyophilized solid is occurring in an essentially linear fashion. If this is not the case, then the amount allowed over time would need to be calculated based on the product's characteristic moisture sorption isotherms.

Leak Rate Specification Test Conditions

Leakage rate specifications should be defined according to clearly stated test conditions of time, pressure, and temperature. For pharmaceutical package systems, it is important to understand that more than one set of standard conditions may apply to even the same product-package. For instance, a virgin multidose parenteral vial is required to prevent all liquid-borne microbial ingress at all conditions of differential pressure anticipated for the product's life over extended periods of time. However, after being punctured at point of use, this same package cannot be expected to survive the same extreme challenge conditions. Such a product would be required to pass different leak rate specifications post puncturing. A similar example is that of a package-device combination system, such as a vial designed with a spiking device intended to allow product access and transfer to an intravenous administration set. Prior to spiking, the vial/closure seal would be required to survive liquid microbial challenges without any product loss over extended times and under all differential pressures representative of product processes or the distribution cycle. After spiking, the package would need to prevent the ingress of airborne microorganisms and ensure complete product transfer. Thus, two very different types of tests and test limit specifications would be needed for the same vial/closure seal. In addition, a package's seal or closure system may need to be evaluated in different ways to ensure that processes do not compromise the

package integrity. For example, a vial may need to be evaluated for its integrity during a terminal steam sterilization cycle, as well as during normal shipping and handling. Finally, different tests and test specifications may be needed to define the integrity of different seals within the same package system. For instance, an induction seal on a liquid-filled bottle may need different specifications and tests than the screw-thread cap covering the bottle finish.

Leak Rate Specification Terminology and Safety Factors

The terms used to define the leak rate specification will differ according to the type of test method used. In some cases, leakage is measured qualitatively, and the specification can be defined in absolute terms. For instance, for a package that is meant to prevent liquid loss and microbial ingress, any detectable liquid leakage under the stated conditions of storage and use would be considered unacceptable. Then, a liquid or microbial ingress leak test specification may be defined as either it does occur (according to the stated conditions) and the package is therefore unacceptable, or it does not occur and the package is considered satisfactory.

On the other hand, when leakage is being measured quantitatively, leakage specifications may be defined in numeric terms. This is the case, for example, when measuring gaseous leakage by helium mass spectrometry, differential pressure, or vacuum loss. Once the leakage rate corresponding to product failure has been defined, a leakage rate limit needs to be established. Given the fact that gaseous leakage rate is a logarithmic phenomenon, and many leakage tests are accurate to only within $\pm 50\%$ of the true value, it is reasonable to use a safety factor when selecting a leakage rate specification. Often, a safety factor of 0.1 is recommended (21). Thus, for a package that must demonstrate 10^{-5} Pa $\cdot$ m^3/s as the maximum allowable leak rate, the leak rate specification would be established as 10^{-6} Pa $\cdot$ m^3/s. When referring to gaseous leakage, the set of standard conditions most commonly accepted

is that of dry air at 25°C for a pressure differential between 1 standard atmosphere and a vacuum (a standard atmosphere is 101.325 kPa). For practical purposes, the vacuum need be no better than 1/100 of an atmosphere or 1 kPa. If test conditions are not specified, these standard conditions are generally assumed (11).

PACKAGE INTEGRITY TEST METHODS

Selection of Equipment

Especially during the development stage of a product-package system, it is important to demonstrate the ability of the package to meet the established leak rate specifications. Therefore, a leak test method should be selected that is capable of measuring at or near the specification rate. For quantitative leakage tests, the leak test instrument or method should be more sensitive by at least a factor of 2 than the minimum leakage to be detected to ensure reliability and reproducibility of measurements. To specify leakage rates lower than necessary to ensure acceptable package performance, or to select a test method capable of measuring leakage rates far smaller than required, is impractical and expensive. The cost of test equipment able to detect a leak of 10^{-3} std cc/s is negligible compared with that having a sensitivity of 10^{-12} std cc/s, which may run 10,000 times higher.

Leak test equipment generally has an optimum window of performance. For instance, bubble testing by immersion in water can accurately detect leakage rates of 10^{-1} to 10^{-3} std cc/s. As leaks approach the lower end of this range, a longer immersion time and perhaps better observation techniques will be required for detection. Leaks just below 10^{-4} std cc/s may be detectable by lengthening the test time, by increasing the pressure differential, or by adding surfactants to the immersion liquid. This modified method may prove impractical, however. On the other hand, measuring leaks above 10^{-1} std cc/s may require steps to ensure that the package does not

experience too rapid a pressure loss before the package can be immersed.

There are other considerations in the selection of leak test equipment, including cost, ease of use, repeatability, testing speed, and safety. Data integrity, storage, analysis, and display capabilities are also important criteria. Selection of leak test methods and equipment should be carefully catered to the package requirements and the needs of the user.

Test Methods

In this section, various package integrity tests useful for the evaluation of parenteral package systems are reviewed. These methods primarily consist of techniques for detecting the presence of a leak or for measuring the degree of leakage. Methods that detect the presence of leaks are called qualitative tests—either the package is found to leak or it does not. Such tests include liquid tracer tests, high-voltage leak detection, microbial challenge tests, or bubble tests. Other tests provide quantitative leak results, such as helium mass spectrometry or some pressure/vacuum decay methods. Then, there are tests that detect the evidence of leakage, such as near-infrared (NIR) spectrometry, which measures the presence of excess moisture in a dry powder product resulting from unacceptable package integrity. Tests such as acoustic imaging or airborne ultrasound may help to visualize a possible leak, or they may serve to better characterize package seals, allowing a more complete understanding of the mechanisms of package integrity. Other tests measure mechanistic characteristics of a closure or seal that may relate to package integrity. The residual seal force test is one example of such an approach.

Each test has advantages to its use and inherent drawbacks. There is no one perfect test for all situations. In many cases, more than one test may be needed. Often, more sensitive test methods that characterize several integrity aspects of a package are required during the earlier development stages of a product. Later, when the package and critical pro-

duction steps have been defined and controlled, less stringent test methods may be more appropriate.

The lists of tests discussed and the suppliers mentioned in this section are not intended to be exhaustive. These are some of the most common methods, but there are likely numerous others in use. The suppliers are those with whom we are most familiar. Ultimately, test methods and instrument supplier selection should be based on a rational understanding of the package design, the critical nature of any potential leaks, and the capabilities of the instrument. The final criteria for selection are ultimately based on the ability of a test method and instrument to reproducibly and reliably identify package integrity failures.

A summary of package integrity test methods; their levels of sensitivity; advantages, and disadvantages; reported usage; and equipment suppliers are presented in Table 4.2. Additional comments on each of these methods are presented at the end of this section.

Acoustic Imaging

Acoustic imaging is a technology used to examine hidden or obscured defects in objects as varied as microchips, construction joints, ceramics, composites, and laminates. In acoustic imaging, specially designed piezoelectric transducers convert electrical impulses into very short ultrasound pulses at frequencies up to several hundred megahertz. An acoustic lens focuses these pulses to a pinpoint that has the power to define tiny features within a sample. After an ultrasound pulse is sent into the sample, the return echoes are detected by a receiver. These arrive at different times, depending on the depth of the feature. A digital waveform on the user interface screen displays the arriving echoes. This echo-time display can be programmed to produce planar images or images representing varying depths within the sample.

This technology has been advertised as a research-and-development tool for evaluating package seals and closure systems of pharmaceutical packaging. While it is a very powerful

Table 4.2 Package Integrity Test Methods

ACOUSTIC IMAGING

Method: Ultrasonic energy is focused into sample submerged in water or other solvent. Echo patterns produce images of package material interior.

Sensitivity: Qualitative imagery

Pros:
- Very sharp image produced
- Structural defects visible, such as channels, delaminations
- Sophisticated tool for package investigations and development

Cons:
- Expensive
- Sample must be immersed
- Slow
- Expertise required
- Not as useful for porous materials

Critical test parameters:
- Package structural design
- Instrument capabilities (transmitters, receivers, imagery analysis)

Reported usage:
- Microchip technology, forensic science, construction materials, packages and devices

Suppliers:
- Sonoscan Inc., www.sonoscan.com

BUBBLE TEST

Method 1: Submerge package in liquid, apply differential pressure, observe for bubbles.

Method 2: Apply surfactant, draw vacuum, and look for foaming.

Sensitivity: $\geq 10^{-5}$ std cc/s
Method dependent; qualitative

Pros:
- Simple
- Inexpensive
- Location of leaks can be observed
- Good early research or troubleshooting technique

Cons:
- Relatively insensitive
- Operator dependent
- Wets package seal; destructive
- Requires gas headspace to be present at leakage site

Critical test parameters:
- Visibility of submerged package (lightning, background, magnification)

Time allowed for inspection
Differential pressure across seal
Submersion fluid or surfactant surface tension
Operator training and ability
Gas headspace present at leakage site
Package cleanliness

Suppliers:
None

Reported usage:
Pipes, large equipment, aerosols (warm water bath test)

GAS TRACER DETECTION

Method: Test tracer gas is used to measure leakage/permeation across a package seal. Gas is detected either by a coulometric detector (O_2) or by a photoelectric sensor (CO_2 or H_2O). Instruments that invasively test package headspace for O_2 or CO_2 are another type of gas detection test method.

Sensitivity: Time/instrument dependent
Quantitative

Pros:
Directly correlates to package performance, protection
Potentially highly sensitive
Provides total leakage and permeation information

Cons:
Slow
Some tests are destructive

Critical test parameters:
Tracer gas detection technology sensitivity and accuracy
Headspace sampling technique (for invasive testing)
Package headspace volume
Concentration of tracer gas inside package

Reported usage:
Screw-cap bottles, food and beverage containers
Blister packages, polymer and foil pouches

Suppliers:
MOCON/Modern Control, Inc., www.mocon.com

Table 4.2 Continued

HELIUM MASS SPECTROMETRY

Method: Helium is used as a tracer gas for detection and measurement of leakage using a mass spectrometer. "Inside-out" method or "sniffer" probe methods are two options when helium is inside the package.

Sensitivity: $\geq 10^{-11}$ std cc/s

Quantitative

Pros:

- Inert gas tracer
- Extremely sensitive test
- Rapid test time
- Correlated to microbial and liquid leakage

Cons:

- May confuse helium diffusion with leakage
- Helium must be added
- May be destructive
- Bombing takes time
- Expertise required
- May not detect large leaks
- Expensive

Critical test parameters:

- Helium concentration inside the package
- Helium sorption onto the package
- Helium background within the test system
- Helium background in surrounding test environment
- Test time duration
- Test chamber size
- Test chamber vacuum level

Suppliers:

- Alcatel Vacuum Technology, www.alcatelvacuum.com
- Inficon, Inc., www.inficon.com
- Varian, Inc., www.varianinc.com

Reported usage:

- Pharmaceutical packaging, refrigeration units, automotive parts, pacemakers, food and beverage containers, drums

HIGH-VOLTAGE LEAK DETECTION (HVLD)

Method: High frequency, high voltage is applied to sealed container. Increase in conductivity correlated to presence of liquid near detectors.

Pros:
- 100% Automatic inspection
- Clean
- Nondestructive
- Rapid

Critical test parameters:
- Conductivity of package and product
- Product proximity to leak
- Package cleanliness
- Humidity of testing site
- Voltage level
- Gain setting

Suppliers:
- Rommelag, www.rommelag.com
- Nikka Densok Limited, www.NikkaDensok.com

Sensitivity: Shown to detect 5-μm holes. Reported by suppliers to detect 0.5-μm defects

Cons:
- Difficult to validate with standard defects
- Liquid-fill product required

Reported usage:
- Glass and plastic ampules or blow/fill/seal containers; glass vials, syringes

LIQUID TRACER TESTS

Method: Package is immersed in solution of a tracer chemical or dye.

Pressure/vacuum or temperature cycling is used to improve sensitivity.

Leakage is detected visually (dye) or instrumentally (dye or chemical).

Pros:
- Correlates to liquid leakage and microbial ingress
- Operator independent (instrumental methods)
- Inexpensive
- Simple to perform

Sensitivity: $\geq 10^{-5}$ std cc/s depends on method, tracer, and detection

Semiquantitative

Cons:
- Destructive
- Human variability (visible dye)
- Probabilistic so larger sample numbers needed
- Slow

Table 4.2 Continued

Critical test parameters for both dye and chemical tests: Immersion exposure time, differential pressure, temperature cycles Volume of liquid in test package Tracer solution concentration, viscosity, surface tension Cleanliness of system and solutions Critical test parameter for dye only: Chemical compatibility with product formulation Human inspection variables (ability, training, environment) Suppliers: Detection instrumentation specific	Reported usage: All types of packages
MICROBIAL CHALLENGE TESTS	
Methods: Containers are media filled, and the seal is challenged with microorganisms (in liquid suspension or aerosol form). Presence of microbial growth is confirmed visually or with instrumentation.	Sensitivity: Erratic, method dependent
Pros: May provide direct correlation to microbial integrity No special equipment required Airborne challenge best approach for tortuous seal tests Critical test parameters for both immersion and airborne tests: Microorganism selected Concentration of challenge organism Growth promotion ability of media inside package Differential pressures, temperatures to maximize ingress Time of exposure	Cons: Insensitive Expensive in time, storage, resources Slow

Critical test parameters for immersion tests:
Viscosity and surface tension of challenge fluid
Presence of liquid path in leak
Critical test parameters for airborne tests:
Challenge vessel design and air circulation patterns
Relative humidity and temperature in challenge chamber
Package position and placement in challenge chamber
Suppliers:
None

Reported usage:
Widely used throughout the pharmaceutical industry

NONINVASIVE MOISTURE AND OXYGEN ANALYSIS

Method 1: Moisture. Near infrared spectroscopy. Used to measure powder moisture inside an unopened glass package.

Sensitivity: 0.2% water content (0.5 mg) in methacholine chloride reported. Calibrated against known standards.

Pros:
Nondestructive to product or package
Rapid (less than 1 minute)
Sensitive to trace moisture
Simple to perform

Cons:
Unknown

Critical test parameters:
Moisture content range within test method sensitivity
Uniformity of test sample moisture

Reported usage:
Lyophilized and dry powder pharmaceuticals, validation of terminal sterilization cycle package integrity

Suppliers:
FOSS NIRSystems, www.foss-nirsystems.com

Method 2: Oxygen and moisture. Tunable diode laser spectroscopy. Laser light is passed through package headspace. Frequency of light matched to oxygen or water. Absorbed light is proportional to headspace contents.

Table 4.2 Continued

Pros:
- Nondestructive to product or package
- Rapid (1 second)

Cons:
- Unknown

Critical test parameters:
- Unknown

Suppliers:
- Lighthouse Instruments, LLC, www.lighthouseinstruments.com

O_2 Sensitivity:
- Test range of 1–100%
- Accuracy ± 0.5% absolute
- Precision ± 0.1% absolute

H_2O sensitivity:
- Test range of 1–100% RH at 25°C to 45°C
- Calibration data available against known standards

Reported usage:
- Products sensitive to moisture or oxygen headspace content

RESIDUAL GAS IONIZATION TEST

Method: High-voltage, high-frequency field is applied to glass vials or bottles sealed under vacuum. The field causes residual gas to glow. Vacuum level is verified by glow or ionization current.

Sensitivity: not documented.
- Qualitative

Pros:
- On-line, nondestructive test
- Rapid

Cons:
- Inconsistencies in results unless parameters carefully controlled

Critical test parameters:
- Vial storage time postsealing
- Voltage and frequency
- Positioning of voltage field and conducting plate
- Package headspace volume
- Package component ionization potential
- Gaseous content of residual headspace
- Package component geometry

Reported usage:
- Lyophilized product

Suppliers:
- Electro-Technic Products, Inc., Chicago, IL

RESIDUAL SEAL FORCE (RSF)

Method: Vials sealed with elastomeric closures are compressed at a constant rate of strain. Stress-strain deformation curves are generated. Second derivative of the curve = RSF value.

Pros:
- Measures closure force post compression
- Nondestructive (although plastic cap may be removed)
- No human error
- Qualitative measurements
- Simple to perform

Critical test parameters:
- Elastomeric formulation, size, history (age, sterilization)
- Design of cap anvil
- Rate of strain
- Load cell sensitivity
- Approach used for detecting RSF second derivative

Suppliers:
- Instron, www.dynatup.com
- Genesis Machinery Products, Inc., www.genmap.con

Sensitivity: Dependent on closures, stress-strain instrument

Cons:
- "Good" RSF will vary with elastomer material and history

Reported usage:
- Parenteral vials (13 to 28 mm)

ULTRASONIC IMAGING

Method: Ultrasound echoes used to create image of heat seals.

Suppliers:
- Packaging Technology & Inspection, LLC, www.ptiusa.com
- Because published information on this technology is not yet available, no further information is offered.

Reported usage: Heat seals

Table 4.2 Continued

VACUUM/PRESSURE DECAY

Method: Change in pressure or vacuum is measured inside the package (destructive) or outside in a sealed package chamber (nondestructive).
Pressure/vacuum change significantly greater than non-leaking package is indicative of a reject.

Pros:
- Clean
- Nondestructive (test chamber method)
- Relevant to package performance (shipping/distribution)
- Relatively inexpensive
- Sensitivity good for leaks $\geq$ 5 μm
- Rapid test (few seconds)

Critical test parameters:
- Rigidity and porosity of package
- Headspace content of package
- Test chamber geometry, volume
- Total volume of internal pressure lines
- Initial chamber vacuum/pressure
- Time to establish initial chamber conditions
- Differential pressure/vacuum decay
- Time allowed for pressure/vacuum decay

Suppliers:
- Packaging Technologies and Inspection, LLC, www.packagingtechnologies.com
- Wilco AG, www.wilco.com
- TM Electronics, Inc., www.tmelectronics.com

Sensitivity: $\geq 10^{-5}$ std cc/s
Time, pressure dependent test.
Quantitative or qualitative use

Cons:
- Difficult to detect leaks $\leq$ 5 μm
- Some package headspace needed

Reported usage:
- Variety of pharmaceutical, food, medical device packages

VISUAL INSPECTION

Method: Look for leaks.

Pros:
- Simple
- Inexpensive

Critical test parameters:
- Quality of inspection area (lighting, magnification, background)
- Time allowed for inspection
- Operator training and skill
- Product characteristics (color, viscosity, surface tension)
- Techniques for leakage acceleration (differential pressure)
- Package uniformity and cleanliness
- Package seal visibility

Suppliers:
- Seidenader Inspection System, www.seidenader.de

Sensitivity: 10^{-1} std cc/s

Cons:
- Insensitive
- Operator dependent
- Qualitative

Reported usage:
- All packages for final inspection, especially parenteral packages such as vials, ampules, syringes

WEIGHT CHANGE

Method: Filled, sealed container is stored at various stress conditions and checked over time for weight loss (liquid contents) or weight gain (dry contents).

Pros:
- Simple
- Directly relates to closure performance
- Quantitative
- Inexpensive

Critical test parameters:
- Volatility or hygroscopic nature of filled product
- Accuracy of scale
- Handling technique
- Sorption by package materials themselves

Suppliers:
- None

Sensitivity: Time dependent, can be excellent

Cons:
- Time consuming test
- Leak location not detected
- Package material sorption may interfere with results

Reported usage:
- Vials (including lyophilized products), aerosols, solid or liquid dosage form packaging

tool that can detect irregularities such as minute defects and even seal delaminations and weaknesses, the technology requires that the sample be submerged in water or other solvent, making it a destructive test. It is also relatively slow, with several seconds to a few minutes required per sample. However, it does appear to be an excellent tool for investigative research on package systems when package integrity is in question.

Bubble Test

Bubble testing is generally performed by submerging the container in liquid, applying a differential pressure, and inspecting for bubbles (Method 1). Alternatively, a surfactant may be applied to the outer surface of a pressurized package that can be inspected for foaming (Method 2). Bubble tests determine the presence and location of leaks that allow sufficient gaseous flow to permit the formation of detectable bubbles in a submersion fluid or in an applied surfactant. The sensitivity of bubble tests may vary anywhere from 10^{-1} to 10^{-5} std cc/s depending on the length of time given for observation, the differential pressure applied, lighting, background contrast, and the surface tension of the immersion liquid. To further improve method reliability and sensitivity, ultrasound detection equipment has been advertised as a tool to detect the foaming noise from subvisible surfactant bubbles. The sensitivity of Method 1 is defined as the number and size of bubbles seen in the submersion fluid in a given period of time for the given test parameters. For Method 2, the sensitivity can be defined as the smallest volume of foam visible.

A disadvantage of bubble tests for parenteral products is the need to wet the package seal, making it impractical for 100% leak detection in the production environment, but useful as a rapid screening test or troubleshooting tool.

In Ref. 17, bubble testing of stoppered parenteral vials was compared to liquid leakage of a chemical tracer solution and to quantitative gaseous leakage rates measured by differential pressure techniques. The bubble test was optimized by

using surfactant in the immersion fluid, a dark inspection background, high-intensity lighting, a 3× magnifying lens, a differential positive pressure of 3 psi inside the vial, and a maximum test time for 15 minutes. Under these special conditions, the bubble test was able to detect leakage rates as low as about 10^{-6} Pa · m^3/s (10^{-5} std cc/s) (Fig. 4.7b).

Gas Tracer Detection

Leak test methods based on detection of gaseous molecules are generally the most sensitive of all leak test methods. Gases such as oxygen, carbon dioxide, and water vapor are commonly used for leak detection of pharmaceutical, food, and cosmetic packages. The sensitivity of all tracer gas detection methods generally depends on the test instrument design and capability and the allotted test time. The type of gas detection system selected is generally dependent on which gaseous moiety is the most critical to package performance. For instance, a carbonated beverage container would likely be evaluated using carbon dioxide test equipment.

Gas tracer methods work by infusing the package with a tracer gas and placing it in a test chamber through which an inert carrier gas (e.g., nitrogen) flows across the outside of the package. Tracer gas migrating out of the package is detected in the gas carrier gas either by a coulometric detector (O_2) or by a photoelectric sensor (CO_2 or H_2O). Such tests are valuable in that they quantitatively measure the permeation and leakage of a gas that may be critical to the stability of the product. Thus, products that are sensitive to oxygen, carbon dioxide, or moisture can be packaged in a system that has demonstrated its ability to provide adequate protection. Although the test takes time to allow for gas migration, the results can be highly sensitive.

Instruments that are designed to pierce a container and test its headspace for O_2 or CO_2 are another type of gas detection test method. Although destructive, this approach is useful for testing oxygen ingress as a function of shelf life for those pharmaceutical products that require an inert gas headspace.

They may also be used to verify the presence of a critical inert headspace in packages as part of manufacturing process and control.

The sensitivity of tracer gas detection devices is based on the ability of the tester to accurately sample and measure gas headspace content in the package. Sensitivity is defined in gas concentration units and can be verified using packages sealed with reference gas mixtures of known concentration.

Helium Mass Spectrometry

Helium mass spectrometry is a highly sensitive tracer gas leak detection technique. In fact, helium molecules are so effective at traversing through pores that helium diffusion through materials may actually be confused with leakage.

When performing helium leak tests, the package is typically charged with helium, and gas detection occurs outside the container in a high-vacuum test chamber ("inside-out" testing). Sweeping a low-vacuum sniffer probe along the outside of the package can also detect leaks. Helium may be added to the package by flooding it prior to sealing, by injecting helium into the sealed package, or by exposing the sealed package to an environment of pressurized helium ("bombing"). Helium testing is rapid (a few seconds for inside-out tests) and can accurately detect leaks as small as 10^{-11} std cc/s.

Kirsch and colleagues, in their investigations of micropipet leaks, selected helium mass spectrometry because of its sensitivity and accuracy. As discussed, the test vials with microleaks embedded in the glass walls were flooded with helium prior to sealing, and helium leak rates were measured for each vial (see "Establishing Leak Rate Specifications"). These vials then served as a model for evaluating the ability of aqueous liquids and liquid-borne microorganisms to traverse a leak. Additional studies were also performed in which these helium-tested vials were used as standards to determine the sensitivity of two differential pressure testing approaches (see "Test Methods, Vacuum/Pressure Decay").

Sensitivity of a helium leak test is measured in gas flow rates of standard cubic centimeters per second (std cc/s) or in helium concentration units of parts per million (ppm). Nationally traceable helium leak standards can be connected to the tester/package chamber system to verify its sensitivity and accuracy. The sensitivity of a specific helium test is based on the ability of the tester to accurately detect helium within the package system. When testing packages completely flooded with helium, the mass spectrometer reading is the same as the package leakage rate. But, if the package contains a diluted concentration of helium, such as when bombing or injecting helium into the headspace, the true package leakage rate will need to be adjusted accordingly.

While helium mass spectrometry is the most sensitive of all leak test methods, there are drawbacks to its use. Because the method is so sensitive, any small amounts of helium in the test system, in the surrounding environment, sorbed onto the package, or permeating through the package can result in inaccurate readings. Helium is rapidly lost through larger leaks, making it difficult at times to detect them. Small package headspace volumes also make leak detection potentially more difficult. Also, not all packages can tolerate the level of vacuum needed for detecting the smallest leaks. Helium must be introduced into the package, making it a destructive test for most pharmaceutical products. While the test is very rapid, bombing processes can take considerable time. And, if the package headspace is not completely filled with helium, calculations must be performed to determine the true leakage rate based on the concentration of helium actually inside the package. This highly sensitive method is also the most expensive technique and requires some training to use it appropriately. However, there is often no substitute for the information this technology can provide.

High-Voltage Leak Detection

High voltage leak detection (HVLD) is commonly used for continuous in-line pinhole inspection of liquid-filled packages

formed from a single, electrically nonconductive material, such as glass ampules or form-fill-seal plastic containers. In HVLD, a high-frequency voltage is applied to the container. Any presence of liquid in or near a leak or possibly a thin area in the wall of the container, will allow increased conductivity and subsequent rejection of the package (Fig. 4.12). This technique is very rapid (thousands of units tested per hour), clean, automated, sensitive (minimum pinhole size claimed is 0.5 μm and is unaffected by package opacity.

One disadvantage of this method is the difficulty in quantitatively validating the unit's sensitivity. Often, pinhole detectors are simply adjusted to cull leakers detected by some other technique, such as the dye immersion test. A creative way of correlating conductivity to HVLD sensitivity and reproducibility was performed by Sandoz AG using test ampules

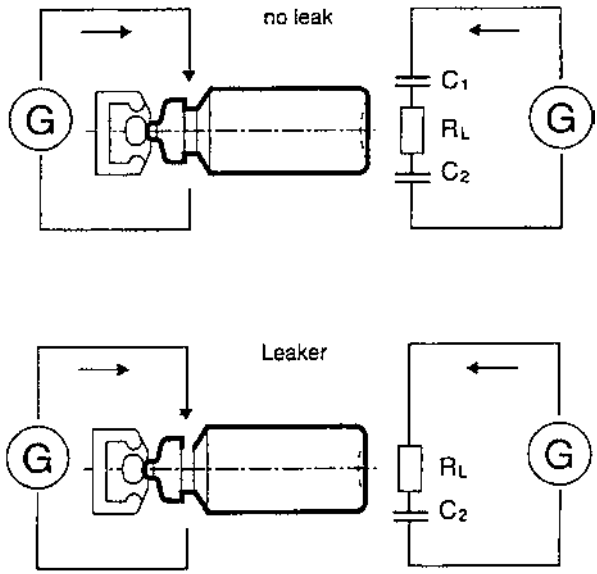


Fig. 4.12 Schematic principle of high-voltage leak detection. G = generator, C_1 and C_2 = condenser; R_L = resistor. (Courtesy of the Parenteral Drug Association, Inc., Bethesda, MD.) (Source: Ref. 24.)

made of Teflon containing defined electrical resistors (25). However, this approach does not allow for correlation to hole size or leakage rate.

More recently, Möll et al. described the validation of an HVLD method for testing low-density polyethylene blow-fill-seal containers filled with a hydrogel-based pharmaceutical product (24). Holes ranging in size from 5 to 30 μm were laser drilled into the walls of the containers. Larger holes of up to 200 μm were manually made using a sewing needle. Once the package's critical testing zones were identified and the appropriate positioning of the detectors was determined, test parameters of voltage and gain were manipulated to establish the optimum conditions for reject detection with a minimum of false-positives (Fig. 4.13). The HVLD was able to detect all pinhole rejects in a population of several hundred test units over three replicate runs. Difficulties reported in this study include the inability to laser drill holes smaller than 5 μm in the plastic and the complication that exposure to high voltage changes the size of the holes.

While HVLD is commonly used for 100% inspection of all glass or plastic containers, this technology is also reportedly being used to check for defects in stoppered vials and prefilled syringes. No literature references on validation of this leak detection method for these packages are currently available.

Liquid Tracer Tests

Using a liquid tracer to detect the presence of a leak is a useful, relatively simple, inexpensive, and potentially sensitive method for detecting defects capable of allowing loss of liquid product or ingress of liquid-borne microorganisms. But, because liquid tracer tests are destructive, they are primarily used for research and development and for production quality control.

The liquid tracer may be either a dye solution detected visually or instrumentally with ultraviolet (UV) spectroscopy or any chemical added to a liquid media detectable by some appropriately sensitive assay method. When performing a typ-

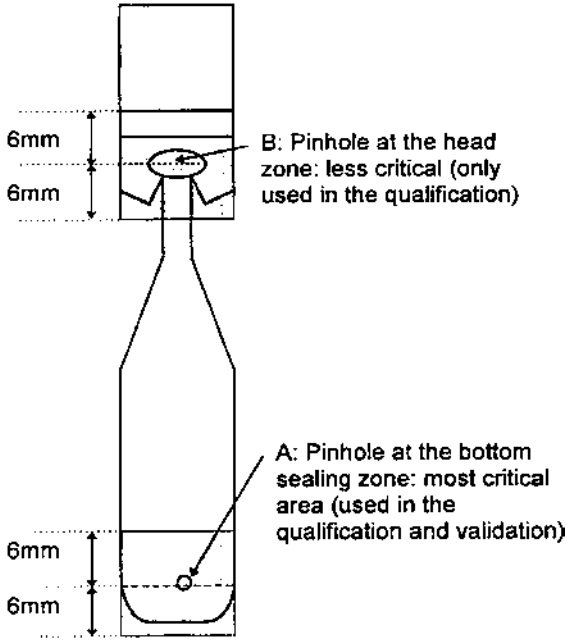


Fig. 4.13 Blow-fill-seal ampule used in the validation of HVLD. The shaded areas represent the areas covered by the two detectors A (at the bottom, or sealing zone) and B (at the top, or head zone). (Courtesy of the Parenteral Drug Association, Inc., Bethesda, MD.) (Source: Ref. 24.)

ical liquid tracer test, the liquid product–filled container is immersed in the liquid tracer solution for a selected period of time. After immersion, the test packages are cleaned and either visually inspected for the presence of dye or the contents are appropriately assayed for tracer chemical. Adding a surfactant or a low-viscosity fluid to increase tracer solution capillary migration can optimize any liquid tracer test. Liquid ingress can also be improved by introducing differential pressures and/or temperature cycling, including autoclaving, during tracer solution immersion. Lengthening exposure time greatly influences test sensitivity (26). It is also recommended

that the tracer liquid be filtered free of extraneous contaminants that might clog any leaks, and that a microbial preservative be added, especially if it is used repeatedly.

Sensitivity of a liquid tracer method is expressed as the minimum volume of tracer solution demonstrated to be detectable in the packaged product. Because detection is dependent on the concentration of tracer in the container, sensitivity can be improved by minimizing the volume of product within the test container. And, because liquid migration through smaller leaks is a probabilistic phenomenon, it is important to test a larger population of test packages when performing liquid tracer tests to minimize risk of false negative results (see "Establishing Leak Rate Specifications, Liquid Leakage Specification").

When using dyes, it is advisable to select a substance that is relatively nontoxic, approved for drug use, chemically and physically color stable in the product to be tested, insensitive to light and heat, rich in color, nonreactive with the product or package, soluble in the cleaning solution, and easily disposed of to meet environmental safety requirements. A typical dye solution formulation may also contain a preservative to prevent microbial growth. Blue dyes are most commonly used, although the human eye more easily detects green to yellow-green. Blue, violet, and red dyes are the colors least easily seen. Visual detection of dye solutions will give variable results depending on packaged product visibility, operator ability, operator training, inspection station design, and inspection time (12). Therefore, care should be taken before relying on dye test results generated solely from human inspection. To eliminate human variability in dye detection, spectrophotometric methods may be used. Jacobus and colleagues reported a spectrophotometric method for Quinizarin Green dye selected for the leak detection of ampules containing an oil-based solution for injection (27).

Chemical tracer tests are potentially more sensitive and semiquantitative than the dye test since detection techniques such as atomic absorption or high-performance liquid chroma-

tography (HPLC) can be used. As discussed, Morton et al. tested simulated parenteral vials for liquid leakage using a copper sulfate tracer solution and an atomic absorption detection assay (17). Kirsch reported the use of magnesium ion as a tracer element to verify the presence of liquid pathways in leaks (22). In both studies, ion tracer test methods were found to be more sensitive and reliable than microbial immersion challenge tests (see "Establishing Leak Rate Specifications, Liquid Leakage Specification").

Microbial Challenge Tests

Microbial challenge tests are performed by filling containers with either growth-supporting product or a culture media, exposing them to microorganisms, incubating the exposed containers at growth-promoting temperatures, and checking them for evidence of microbial growth. This approach to validating package integrity is extensively used by the pharmaceutical industry in attempts to directly correlate defects to microbial contamination risk.

Unfortunately, microbial challenge tests may yield erratic results that do not reliably correlate to leak size or presence. The research to support this was discussed in the section, "Establishing Leak Rate Specifications." Anyone who has conducted microbial ingress tests knows that they can be tedious and difficult to perform. Because it has been shown that even significant leak pathways will not always demonstrate microbial leakage, a large database of samples is needed to minimize the risk of false-negative results. Whenever possible, one should consider tests that more accurately predict the presence of critical liquid leakage, such physical-chemical package integrity tests or related package assembly and performance assessments. However, there are situations when it is valuable to determine the ability of a package design or seal to prevent actual microbial ingress. This is especially true when attempting to validate the design of a tortuous path barrier intended to prevent the ingress of airborne microorganisms.

There are three basic variations to microbial challenge tests (9,28). *Microbial immersion tests* challenge the container by immersion in an aqueous microbial suspension. *Microbial aerosol tests* utilize an aerosolized microbial suspension challenge. With either the immersion or aerosol tests, a dynamic stress of vacuum and/or pressure cycling may be used. The third type of test is called the *static ambient test*. It involves the storage of packaged media or product under typical warehouse conditions—the stored containers are checked for evidence of microbial ingress over time.

For each of these basic tests, there are many variations in use, some of which are described more fully below. Test options should be carefully selected based on the package and product being tested and on the expected environmental and processing challenges to be faced by the product/package system. For all tests, it is important to include positive control test packages to demonstrate the ability of the test to detect at least some of the package leaks of concern. Because microbial challenge tests are not 100% effective in detecting leaks, the test package population should be significantly large to be sure package integrity problems are identified. Microbial challenge test method sensitivity is described in terms of the ability of the test to detect the known positive control samples.

Immersion Tests Very simply, the immersion test involves terminally sterilizing broth- or product-filled packages, followed by immersion of the entire package or the seal area in an aqueous suspension of microorganisms for an extended period of time. Alternatively, packages may be used that have been aseptically filled with media and checked for growth prior to microbial challenge (Fig. 4.14) (28). The packages are then incubated, and nonsterile containers are visually identified. Often, the microbial identity of any positive samples is confirmed to screen out any false positives.

In designing an immersion test, it should be remembered that terminal sterilization may change the nature of some types of package seals by, for example, relaxation of elasto-

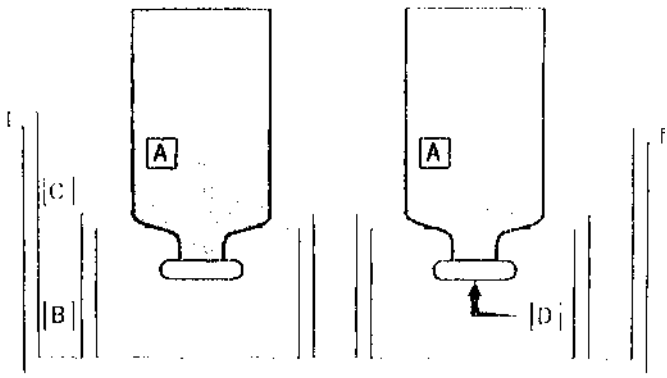


Fig. 4.14 Container/closure system microbiological immersion test. (Courtesy of Interpharm Press, Buffalo, Grove, IL.) (Source: Ref. 28.)

mers or distortion of thermoplastic materials. Therefore, if possible, the sterilization cycle should duplicate the one actually used for the product. When validating packages intended for aseptically filled products, it would be best to use aseptically filled media test units. Otherwise, it is important to verify that the seals or closures of the terminally sterilized test packages have not been impacted by the sterilization treatment.

An additional test variable to consider is the selection of the challenge microorganism. Examples of those reportedly used for such tests include *P. aeruginosa* (17,28), *Pseudomonas diminuta* (*Brevundimonas diminuta*) (19), *Enterobacter coli* (19), and *Serratia marcescens* (29). Size, motility, and viability in the product or culture media are all factors in microorganism selection. To maximize chance of success, the microbial challenge concentration should be at least 10^5 counts per milliliter at test completion. There are various culture media that may be used depending on the nature of the challenge microorganism. Vacuum and/or pressure cycles may be in-

cluded in the challenge test to further stress the package or to mimic anticipated product processing, distribution, and storage conditions. Incubation temperatures should be selected that are most appropriate for growth of the challenge microorganism. In some cases, consideration may be given to venting the package closure with a sterile needle containing a vent filter if an obligate aerobic microorganism is used in the test (28). Microbial growth, as evidenced by cloudiness in the package, may be detected visually or with instrumentation. In the case of product-filled packages, verification of nonsterility may require aseptic filtration and filter plating for microorganism identification. Any nonsterile package contaminants are generally identified to verify the challenge microorganism as the source of contamination.

As discussed in "Establishing Leak Rate Specifications," the sensitivity of microbial immersion challenge tests has been compared in recent years to that of a bubble test, liquid tracer tests, helium leakage tests, and pressure decay tests. These results by researchers working with Kirsch and Morton all support the following conclusions: (a) liquid-borne microbial ingress is dependent on the presence of a liquid pathway; (b) ingress is not assured even when liquid pathways are present; (c) ingress is a probabilistic phenomenon, with greatest probability of ingress for larger leaks (greater than about log -5 std cc/s).

Aerosol Tests Packages evaluated by the aerosol test are placed inside a confined vessel or chamber, in which they are challenged with a nebulized cloud of microorganisms at concentrations of about 10^3 microbes per cubic foot (Fig. 4.15). Pressure dynamics may be incorporated to simulate anticipated product shipping or processing conditions or simply to optimize microbial ingress. This is a less stringent and less sensitive test than the immersion test, primarily because there is no liquid present to provide a means for organism ingress, and the concentration of challenge organisms is significantly less. However, an aerosol challenge may be a more real-

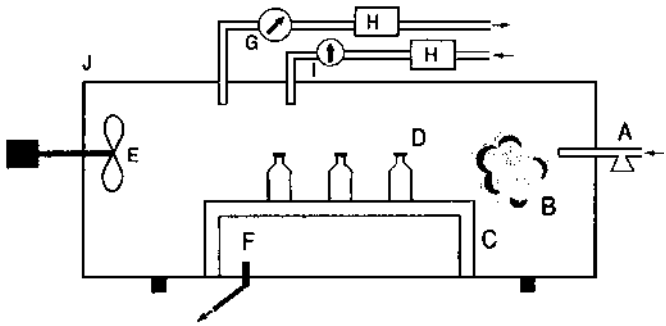


Fig. 4.15 Microbial aerosol test system to evaluate container closure systems. (Courtesy of Interpharm Press, Buffalo Grove, IL.) (Source: Ref. 28.)

istic test for those packages that contains no fluid and rarely, if ever, are wetted during manufacture or product life.

Considerations is designing an appropriate aerosol challenge test include vessel design and package position and placement in the chamber, all of which can affect the uniformity of the microbial suspension. The environmental conditions of humidity and temperature should be selected to ensure the viability of the microorganisms. In some cases, the surfaces being tested may be coated with agar, broth, or a diluent such as glycerol to prevent impacted microbes from becoming desiccated. An excellent review of aerosol challenge tests along with cited references of original research can be found in Ref. 28.

Static Ambient Tests Static ambient tests involve placing the product of media-filled packages in storage and evaluating them over time for sterility. This type of test may utilize packaged culture media taken from the filling validation run for an aseptically filled product. Product stored and tested at ex-

piry for sterility is also a type of static ambient test. Actual or simulated shipping tests of the filled package may be included as part of the test. Because there is no concentrated microbial challenge to the product, this type of test is the least effective at detecting leakage. Therefore, static ambient tests are not recommended unless there is a specific justification to support their use.

Noninvasive Moisture and Oxygen Analysis

Interest has been building in recent years in two relatively new methods of noninvasive analysis of packaged product moisture and oxygen content: Near Infra-Red spectroscopy (NIR) and tunable diode laser spectroscopy (TDLS).

In NIR spectrometry, the NIR spectrometer is operated in the reflectance mode to test the contents of a closed glass container. Because the water O—H band shows a strong signal in the NIR region of the electromagnetic spectrum, low levels of moisture in the package contents can be measured. The test may be used qualitatively, or the measurements can be calibrated and used quantitatively. Reported to be rapid (less than 1 minute per test) and simple to use, this method shows promise as a tool for evaluating the package integrity of dry powder and lyophilized products.

In 1993, Last and Prebble reported the use of NIR for lyophilized product moisture evaluation (30). More recently, Birrer et al. used NIR as a way to verify the package integrity of parenteral vials during a terminal steam sterilization cycle. The test vials were each filled with 250 mg of methacholine chloride powder prior to steam exposure. At the end of the cycle, this highly hygroscopic powder was visually examined for evidence of liquefaction and was tested by NIR. NIR was found to be more sensitive than visual inspection and was able to detect as little as 0.5 mg of water, or 0.2% of the total powder content (31).

TDLS is another, even newer, method for nondestructively testing packages for relative humidity, as well as oxygen content in the package headspace. This technology, marketed

by Lighthouse Instruments, LLC, works by shining a laser light through the container headspace and tuning the laser frequency to match the internal absorption frequency of the molecule being analyzed. Readings are calibrated against known standards for quantitative reliability. It is reported that either relative humidity or oxygen content in the range of 1% to 100% can be measured in a single second (32).

Residual Gas Ionization Test

The residual gas ionization test (also called the vacuum ionization test or the spark-coil test) by Electro-Technics Products, Incorporated, has been cited in the literature as a tool for verifying the presence of vacuum in vials or bottles (33). A high-voltage, high-frequency field is applied to the bottles or vials, which causes ionization of the residual headspace gas. As ions and electrons recombine, visible light is observed. Observation can be made manually or with the aid of a conducting plate located near the bottle wall that measures the level of ionization current. Measurable current or visible light is indicative of the loss of vacuum within the bottles. The sensitivity of this method is based on its ability to accurately and reliably detect pressure exceeding a specified acceptance limit in evacuated packages. Control packages sealed under known levels of partial pressure should be used for sensitivity and validation studies. Sensitivity is defined as the range of partial pressure readings that can be detected as a function of voltage, frequency, the package, and the test package system configuration. While this is a nondestructive, rapid test that potentially permits 100% batch testing, residual gas ionization does have its drawbacks. Namely, it is essentially a qualitative test and care must be taken to control all test parameters to prevent inconsistencies in test results (34,35).

Residual Seal Force

The parenteral vial serves as a useful illustration of how package assembly and processing variables correlate to package

integrity. Effective sealing of vials is a function of the dimensions of the package components and the capping operation. Controlling package component dimensions will ensure consistent "stack height" of the vial plus the closure relative to the aluminum seal skirt length. A longer aluminum seal than necessary, or a shorter stacking height, will result in a loosely crimped vial. Alternatively, a seal skirt too short or a taller stacking height may result in a poor seal due to closure dimpling.

Elastomeric closures crimped to the finish of parenteral vials are subject to capping machine head pressure and crimping force, which act to compress and hold the stopper onto the top of the vial. The compressive force the closure continues to exert onto the vial finish after sealing ensures package integrity. This force is termed the *residual seal force* (RSF). Because closures are viscoelastic in nature, the RSF will decrease as a function of time, processing procedures, and elastomer composition.

RSF values can be determined using a constant rate of strain stress tester, also called a Universal Tester, such as manufactured by Instron. Morton and Lordi first reported the use of a Universal Tester to measure RSF values in the late 1980s (36). Alternatively, Genesis Machinery Products, Incorporated, has recently introduced an Automated Residual Seal Force Tester (ARSFT) that works according to the same principle. To perform an RSF test, a specially designed aluminum cap is placed on top of the sealed vial and placed on a compression load cell of the Universal Tester or ARSFT. The vial is then slowly compressed at a consant rate of strain, and a stress-deformation response curve is generated. The RSF is the force at which the slope of the curve demonstrates a noticeable decrease.

Ludwig et al. worked to optimize the RSF method by modifying the metal cap anvil that is placed on top of the vial (37,38). Rounding the top of the metal cap anvil helped to make a more uniform compression of slightly imperfect vials. Making the cap fit more tightly helped improve centering of

the cap anvil onto the vial (Fig. 4.16). In addition, Ludwig and coworkers developed a program that automatically collected the RSF value from the second derivative of the strain-strain deformation curve. Both 13-mm and 20-mm finish vials were tested by this technique. Figure 4.17 illustrates an ideal RSF curve. Stress deformation data produced from five replicate readings of a single vial using an optimized cap anvil are shown in Fig. 4.18.

Morton and Lordi published data to demonstrate the usefulness of the RSF method (39). Figure 4.19a shows the range of RSF values seen among three lots of the same formulation. Figures 4.19b and 4.19c demonstrate how factors such as time and terminal steam sterilization can change RSF values. Because residual seal force is a direct indication of how tightly a parenteral vial is sealed, it was shown to be possible to correlate RSF to leakage rate. First, closures of various elastomeric materials were applied to test vials at a range of seal forces. Some of the vials had been etched with channel defects across the finish. For each closure/compression seal combination, the leakage rate was measured using a differential pressure tester, and the residual seal force was measured. A vial was considered "sealed" once the vial's leakage rate dropped below the leak detector's lower limit of detection. As illustrated in Fig. 4.20, leakage was correlated to greater defect depth on the vial finish area, lower closure compression force, and lower RSF. Different amounts of closure compression was required to seal a given defect, depending on the viscoelastic nature of the closure material. (For additional information on this differential pressure test, see "Test Methods, Vacuum/Pressure Decay.")

Finally, a few comments are offered about RSF techniques. It is our experience that RSF tests are best performed with only the aluminum seal portion of the cap, without any added plastic top. As demonstrated by Ludwig et al., the strain rate must be optimized according to the size of the closure, with 13-mm closures requiring a lower rate of strain than 20-mm closures. Sensitivity is measured in units of force; the degree of sensitivity will be a function of the accuracy of the in-

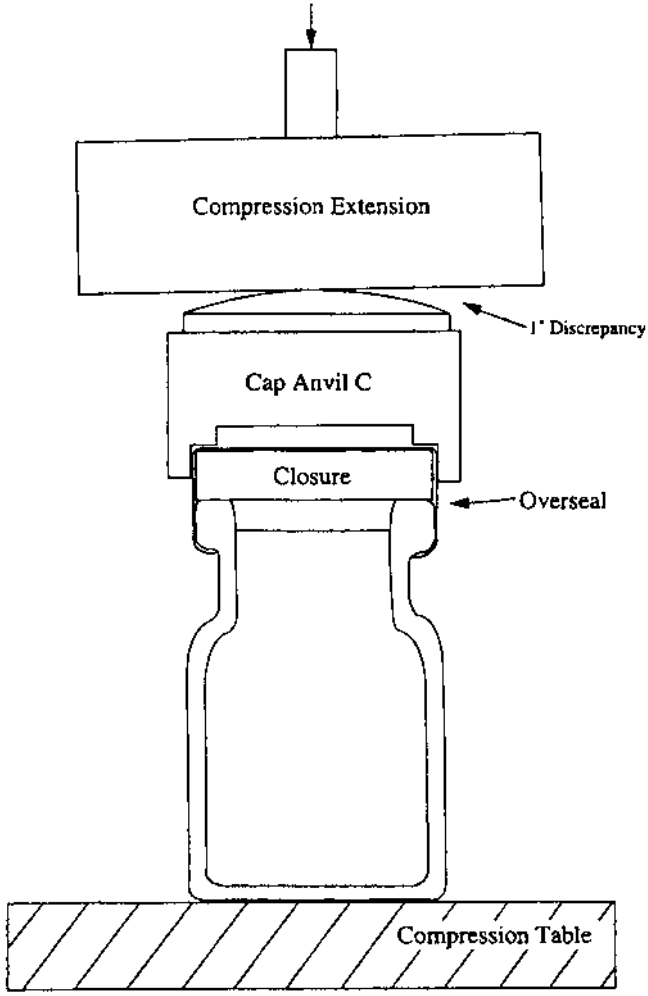


Fig. 4.16 Residual seal force technique. Ludwig-modified cap anvil positioned on parenteral vial. (Courtesy of the Parenteral Drug Association, Inc., Bethesda, MD.) (Source: Ref. 37.)

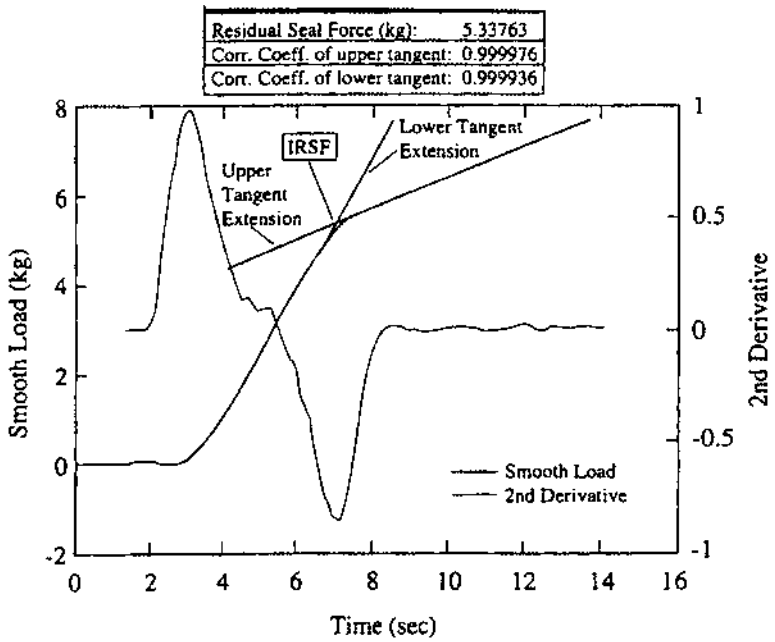


Fig. 4.17 Stress-deformation plot of smooth load and second derivative versus time showing the final output from the RSF determination macro. The upper and lower tangent lines are drawn, and the Instron residual seal force (IRSF) and regression correlation coefficients are located in the data box. (Courtesy of the Parenteral Drug Association, Inc., Bethesda, MD.) (Source: Ref. 37.)

strument and the error inherent in stress-strain readings. The deviation in RSF values can be determined by compiling the error seen among a population of vials sealed with a given elastomeric closure formulation, over a range of sealing forces, among operators, and between instruments. It has been our experience, supported by research by Ludwig et al., that optimized residual seal force tests can provide very reliable information on the quality of parenteral vial compression seals.

Ultrasonic Imaging

Ultrasonic imaging is a new technology currently available through Packaging Technologies and Inspection, LLC. (PTI).

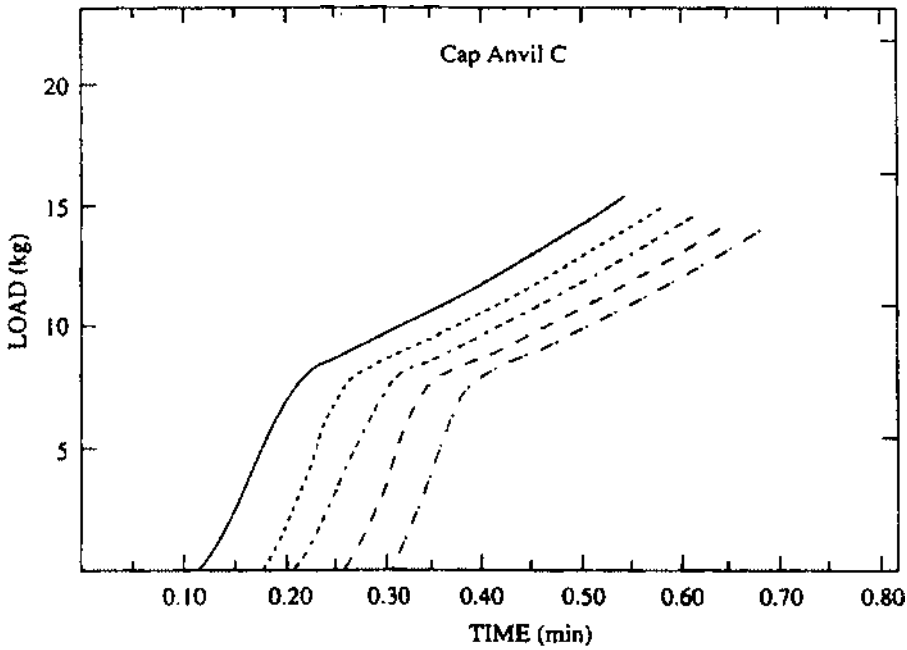


Fig. 4.18 Stress deformation data produced from five compressions of a single vial using Ludwig-modified cap anvil C. The test vial was sealed with a 20-mm diameter Helvoet 6207 lyophilization closure. Plots were separated on the x-axis for clarity of presentation. (Courtesy of the Parenteral Drug Association, Inc., Bethesda, MD.) (Source: Ref. 37.)

This technique is unusual in that ultrasonic imagery of a package or its materials can be performed without immersing the package in a conducting medium such as water. Novel transducers are used to allow extremely high transmission of ultrasonic energy through air and other gaseous media. Reflected and transmitted ultrasonic signals are used to generate surface and internal images of a package that allow the detection of internal discontinuities, the examination of microstructures, and a comparison of material characteristics. To date, airborne ultrasound has been found to be useful for testing heat seals for the presence of contaminants, leaks, and seal

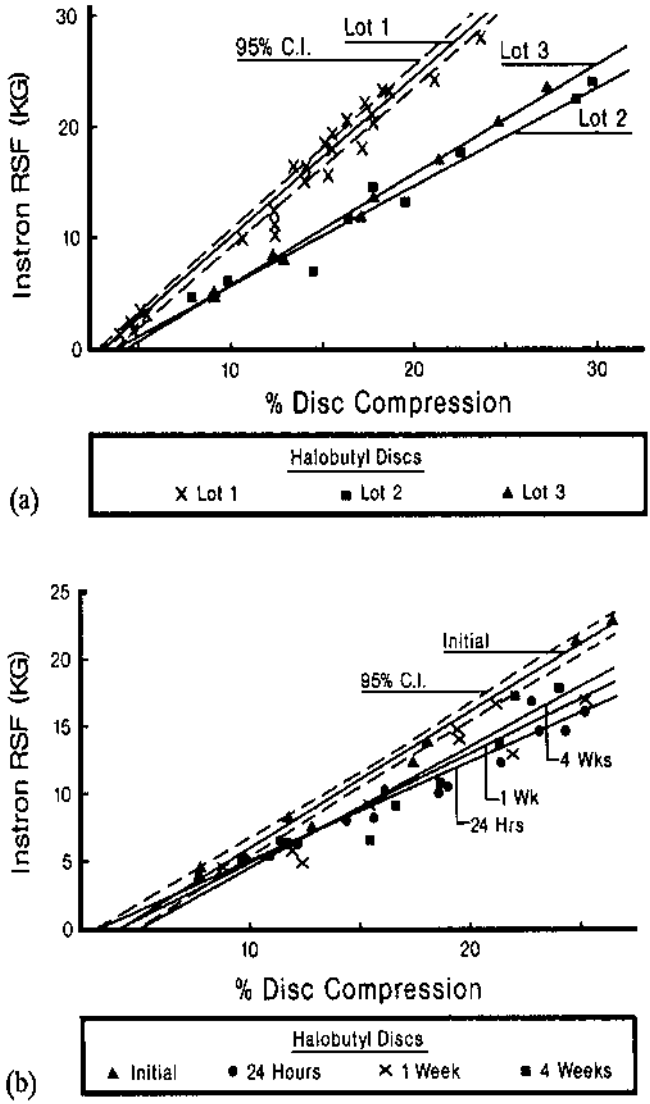
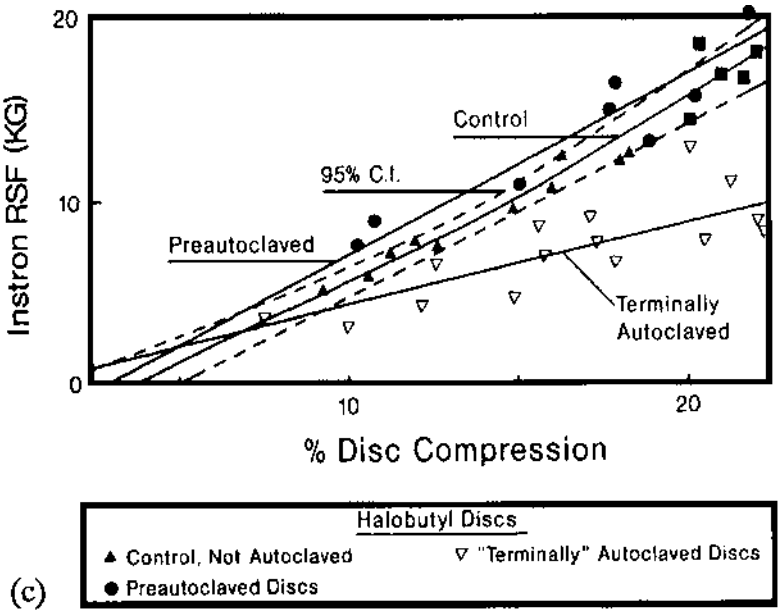


Fig. 4.19 Residual seal force (RSF) test results. (a) RSF closure lot-to-lot differences; (b) time-after-capping effects on RSF; (c) terminal steam sterilization effects on RSF. (Courtesy of the Parenteral Drug Association, Inc., Bethesda, MD.) (Source: Ref. 39.)



weaknesses, and for structural density. Once this technology becomes fully developed, it should be useful as a nondestructive tool for research, investigations, and production quality control of a variety of package seal/closure surfaces.

Vacuum / Pressure Decay

Vacuum or pressure decay leak tests are often used for many package applications, including food, medical device, and pharmaceutical packaging. One major advantage such tests offer is they can usually be performed on the packaged product as is; the package is not contaminated by the addition of dyes, chemicals, or gases, and it usually is not physically damaged. The principal behind this technique is to place the package in a tightly closed test chamber, exert a pressure or vacuum inside the chamber, and then measure the rate of pressure/vacuum change in the chamber over time. The rate or extent of change is compared to that previously exhibited by a control,

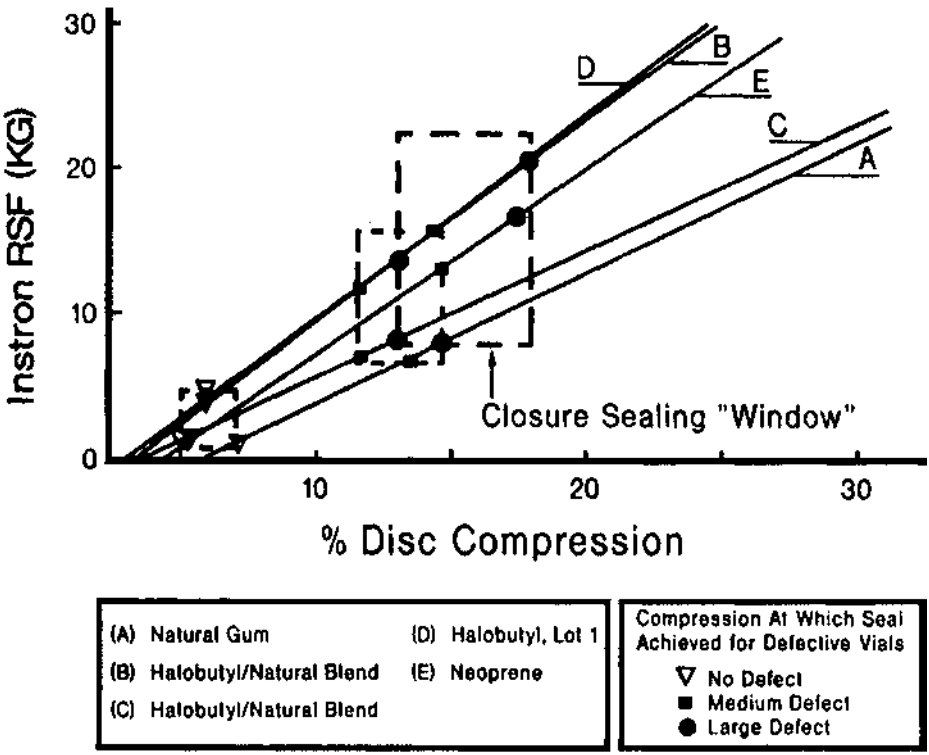


Fig. 4.20 Leakage versus percentage closure compression on simulated parenteral vials. Sealing ability of closures compared for vials without defect versus those with channel defects etched into the vial finish. (Courtesy of the Parenteral Drug Association, Inc., Bethesda, MD.) (Source: Ref. 39.)

nonleaking package. Significantly greater change for a test package is indicative of a leak.

As described in the section, "Establishing Leak Rate Specifications, Liquid-borne Microbial Leakage Specification," Morton et al. used the concept of pressure change to evaluate the sealing ability of elastomeric vial closures. A differential pressure transducer attached to a test manifold was used to measure the leakage from simulated test vials stoppered with

various elastomeric closures (Fig. 4.4) (16). With this device, stoppered vial leakage rates between 10^{-3} and 10^{-7} Pa · m^3/s (10^{-2} and 10^{-6} std cc/s) were quantitatively measured. The linear regressions in Fig. 4.20 represent the percentage the closure flange was compressed as a result of capping the (x-axis), versus the residual seal force value (y-axis) for vials stoppered with closures of differing viscoelastic properties. The "windows" in this figure indicate the minimum amount of closure compression required to seal defective test vials. The defects consisted of channels of differing depths etched across the sealing surface. A vial was considered "sealed" once the vial's leakage rate dropped below the leak detector's lower limit of detection. As illustrated in the figure, leakage was correlated to greater defect depth on the vial finish area and lower compression force of the closure onto the vial. Different amounts of closure compression were required to seal a given defect depending on the viscoelastic nature of the closure material. (For an explanation of residual seal force, see "Test Methods, Residual Seal Force."

One type of vacuum/pressure test is the vacuum retention test. To perform this test, samples from a population of packages sealed under vacuum are checked for the presence of vacuum over time. Vacuum measurement is made using a vacuum gauge connected to a needle for puncturing the package.

Today, pressure/vacuum decay instruments are commercially available for nondestructively testing all types of pharmaceutical packages. Packaging Technologies and Inspection, LLC, TM Electronics, Incorporated; and Wilco AG all produce instruments geared toward parenteral package evaluation for laboratory and production use. In each case, the package is placed in a test chamber, and a vacuum or positive pressure is established within the chamber. The test chamber pressure is monitored for any change resulting from package leakage within a programmed test time. If the pressure change exceeds the previously established baseline for nonleaking packages, the container is rejected. When selecting a test system,

there are several factors that should be considered. The sensitivity and reliability of the instrument's differential or absolute pressure transducer and the capabilities of the internal pressure/vacuum generator are two critical considerations. The test chamber and all internal pressure lines should be of minimal volume for maximum pressure change sensitivity, and they should be well constructed to reduce risk of background leaks. Chamber designs are available from suppliers to restrain nonrigid packages, allowing testing of flexible pouches, bags, and lidded cups. Instruments are available that have a dual-test method option that permits rejection of gross leaks prior to testing for smaller leaks. Instruments should also be compared for their ability to offer any diagnostic test information and data analysis features. The last consideration is, of course, the ability of the instrument to reproducibly and reliably detect defects for a given package type.

When validating an instrument for test method sensitivity, it is important to develop and optimize an appropriate test method. Several critical parameters can be manipulated in test method development. The first is initial chamber target vacuum or pressure. More rigid, nonporous packages can tolerate a greater differential pressure, allowing for a more sensitive method. Other parameters include time to achieve chamber vacuum/pressure, extent of differential pressure/vacuum change, and time allowed for pressure/vacuum change. Manipulation of these variables will enable the establishment of the most optimum method for a given package. Testing known good packages and comparing the results to a population of leakers then completes instrument sensitivity validation. Leakage can be accomplished by using actual packages with typical known defects, by using packages that have been modified with controlled defects (such as the Wilco research reported below), or by introducing a calibrated standard peak rate into the instrument's test chamber. Sensitivity is defined as the largest hole, defect, or flow of gas detectable using the defined test method.

To determine the sensitivity of commercially available vacuum/pressure decay instruments, two published research studies were performed using instruments manufactured by Wilco AG. The first study tested for leakage from empty vials (40), and the second used solvent-filled vials (41), so that pressure changes resulting from either leakage of package headspace gases or from vaporization of package liquid contents could be measured. In both studies, test vials with micropipet leaks in the glass walls were used, ranging from 0.1 to 10.0 μm in diameter. These test vials were identical to those employed by Kirsch and colleagues from helium, liquid ingress, and microbial challenge leak tests (see Fig. 4.8 and "Establishing Leak Rate Specifications"). Each test vial was measured for quantitative leakage by helium mass spectrometry both before and after differential pressure testing to verify the size of the leak present. For the Wilco Liquid Filled Container (LFC) test method, pharmaceutically relevant aqueous solvents were used to determine the impact of solution vapor pressure on leak detection.

Results showed that the standard vacuum decay test varied in its ability to detect leaks depending on the length of time allowed for vacuum decay. The data in Table 4.3 indicate that the practical limit of sensitivity for this technique and

Table 4.3 Estimated Limit of Detection for the Vacuum Decay Test in Terms of Helium Leak Rate and Nominal Pore Diameter

Vacuum test time (s)	Detection limit (std cc/s)	Corresponding nominal pore diameter (μm)
5	1.1×10^{-3}	2
10	8.8×10^{-4}	2
60	4.2×10^{-4}	1
900	6.6×10^{-5}	0.6

Source: Ref. 40.

instrument using empty, helium-filled vials is between log −3 and −4 std cc/s (40).

The LFC results showed about a 14-fold improvement in leak detection sensitivity. For the LFC method, the practical minimum detectable leak was associated with a leak rate of 5×10^{-4} to 6×10^{-4} std cc/s, which corresponds to a leak size of between 1 and 2 μm. Figure 4.21 shows a direct correlation among LFC, standard vacuum decay leak test methods, and helium leak rates. Interestingly, the relationship between LFC response and leak rate did not vary greatly with solvent.

Because both studies used vials that had also been tested for helium leak rate, and because helium leak rate for these packages had been previously correlated to the probability of liquid microbial ingress (see Ref. 19 and "Establishing Leak Rate Specifications"), an indirect correlation could therefore be made between these vacuum decay methods and microbial ingress. Figure 4.22 illustrates the relationship between the probability of microbial ingress and LFC measurements. The

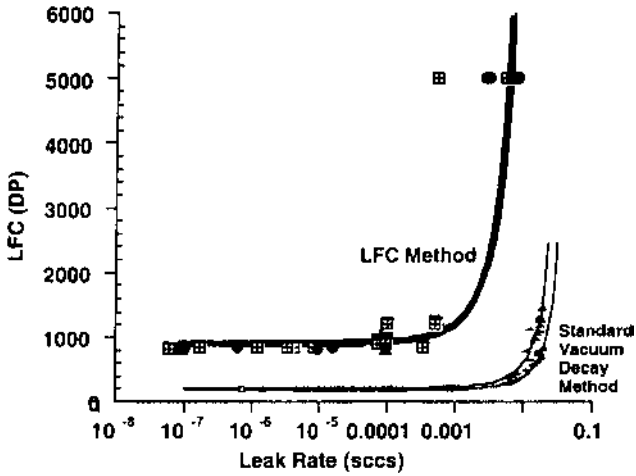


Fig. 4.21 Direct correlation between Wilco LFC, standard vacuum decay leak test methods, and helium leak rates. (Courtesy of the Parenteral Drug Association, Inc., Bethesda, MD.) (Source: Ref. 41.)

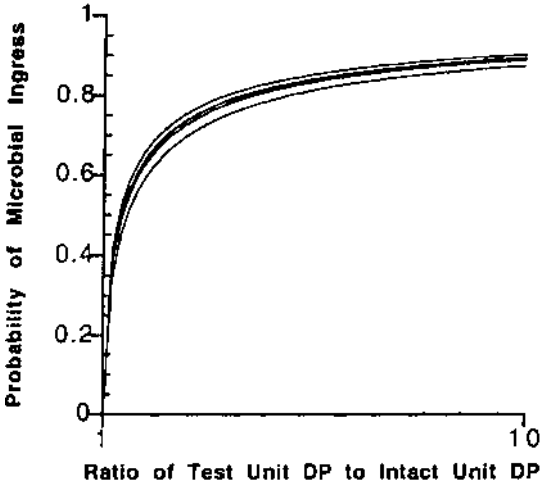


Fig. 4.22 Derived relationship between the probability of microbial ingress and Wilco LFC vacuum decay leak test method. (Courtesy of the Parenteral Drug Association, Inc., Bethesda, MD.) (Source: Ref. 41.)

effective detection range for the LFC method (i.e., when the ratio of test unit DP to intact unit DP is significantly greater than 1) corresponds to a leak size region associated with 40% to 100% chance of microbial ingress.

In conclusion, because of the time factor required for package testing, today's vacuum/pressure decay instruments can serve as a reliable check for larger leaks (greater than about 10 μm) when used in support of production line operations. However, when test time can be extended, such as in research and development or during quality control testing, detection of smaller leaks (less than 2 μm) is certainly possible.

Visual Inspection

Visual inspection is certainly the easiest leak test method to perform, but it is also the least sensitive. Visual inspection is essentially a qualitative method useful for detecting only

larger leaks. Visual inspection can be improved with the use of magnification and special lighting and background. Exposing the package to differential pressure is another way to accelerate potential leakage. When attempting to validate such a method, sensitivity is defined as the incidence of failures detected in a mixed population of positive control leakers as well as good packages. Positive controls should include leaks with sizes as close as possible to the smallest leaks of concern thought to be detectable by this approach. Because visible inspection techniques are considered relatively unreliable and insensitive, they are not recommended as the sole leak detection method for any package application. However, despite all precautions, package failures do occur that are visibly evident, making visual inspection of products and packages a useful part of the inspection and testing of any product-package system.

Weight Change

To perform weight change tests, a population of filled and sealed containers is monitored for weight loss (for liquid-filled packages) or weight gain (for dry-filled packages) over time. Test conditions may include container storage at extremes of relative humidity and/or temperature. Weight change tests are required package integrity test methods in the U.S. Pharmacopeia (USP) for unit-dose and multiple-dose packages for solid oral dosage forms, as well as for bottles intended for liquids (42,43). Weight gain/loss tests are quite accurate and simple to perform and provide useful information that directly correlates to packaged product performance.

Test method sensitivity is defined as the smallest weight change that can be reliably detected given the sensitivity of the scale, the weight and the moisture sorption tendencies of the package, and the variability of the testing and handling techniques used. In some situations, control packages containing inert materials are useful comparisons for identifying significant weight changes in test packages. It is for this reason that the USP method for testing plastic bottles for weight

gain includes a comparison of desiccant-filled bottles to glass bead–filled bottles (42).

Daukas and Trappler reported test results in 1999 that showed how vacuum loss in lyophilized vials can be quantitatively and nondestructively measured quite accurately by monitoring the weight gain of vials after sealing (35). Slow leakage and loss of vacuum were evidenced by a significantly greater weight gain for compromised vials compared to control vials. A correlation in results was demonstrated for vials tested by both weight gain and by a residual gas ionization technique (by Electro-Technic Products, Inc.).

One drawback of weight change tests is the extended time required to allow for the change to occur. Weight loss studies can be expedited by storing the packages at elevated temperatures or low humidity or by filling the containers with materials that more rapidly lose weight. For instance, dry ice has been used in weight loss tests of intravenous screw-cap bottles (44), and isopropanol has been filled into parenteral vials stoppered with closures at various compression levels (9). Conversely, weight gain of dry solids or powders, including lyophilized products, can be accelerated by exposure to high-humidity conditions. In such cases, the package being tested should be allowed to equilibrate to ambient temperature in a dry atmosphere prior to reweighing to prevent error in weight determinations from moisture sorption to the packaging materials themselves.

THE CHANGING PHARMACEUTICAL INDUSTRY

The pharmaceutical industry has grown familiar with traditional parenteral dosage from package systems. Glass ampules, stoppered glass vials or bottles, plastic ophthalmic solution dropper-tip bottles, and prefilled syringes have been successfully manufactured for decades. Such “simple” package systems are typically thought to have well-understood sealing mechanisms, so some have felt that the integrity of these systems has been validated through years of successful manufac-

turing experience. However, every year package recalls continue to be reported due to package integrity failure and leakage from even conventional packages.

The continual challenge to provide integral packaging is now complicated by the growing demand for novel, more complex package systems. Parenteral packages are being designed for easier preparation and safer administration. The ADD-Vantage® vial/flexible container admixture system by Abbott Laboratories (Fig. 4.23), the Heparin Dextrose double-bag product by Baxter Healthcare (Fig. 4.24), and the Vetter Lyo-Ject® dual chamber syringe designed to contain both the in situ lyophilized product and diluent (Fig. 4.25) are all early examples of this approach to packaging. Newer packages now on the market include the Inter-Vial®and Vari-Vial® systems by Duoject Medical Systems, Incorporated (Fig. 4.26), the Clip'n'Ject reconstitution system for use with lyophilized pharmaceuticals by West Pharmaceutical Services (Fig. 4.27), the Bio-Set™ transfer devices by Baxter Healthcare Corporation (Fig. 4.28), and even form-fill-seal packages that can be used for unit-dose intravenous line flushes, like the Viringe™, (formerly known as the Vasceze™) by Avitro, Inc. (Fig. 4.29). Not only are newer package designs available, but also packaging materials themselves have continued to evolve. For example, elastometric closures can be coated with more inert, lower particulate shedding polymeric materials, and less reactive, sterilization-compatible plastics have been commercialized for vials, bottles, and flexible containers.

Sterilization method changes have also taken place over the last 10 to 15 years that influence package selection and design. The typical techniques for sterilizing either package components or packaged product using dry heat, steam, ethylene oxide or gamma irradiation are still widely used. But, advances in steam sterilization technology allow for pressurized cycles that can accommodate packages prior to "blow-out," as well as higher temperature/more rapid cycles that permit sterilization of marginally thermal-stable chemical com-

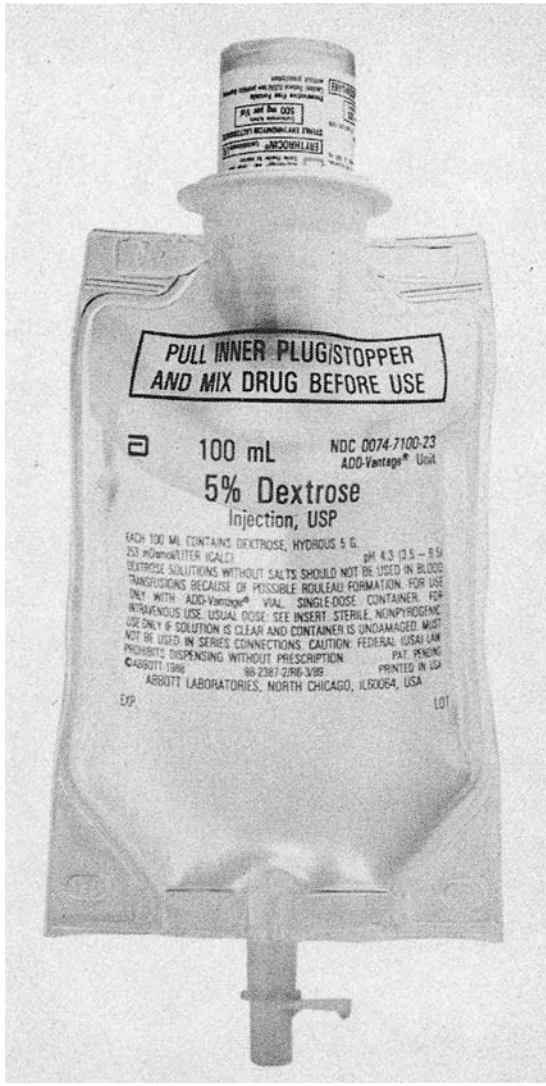


Fig. 4.23 Abbott ADD-Vantage package system. (Courtesy of Abbott Labs, North Chicago, IL.)

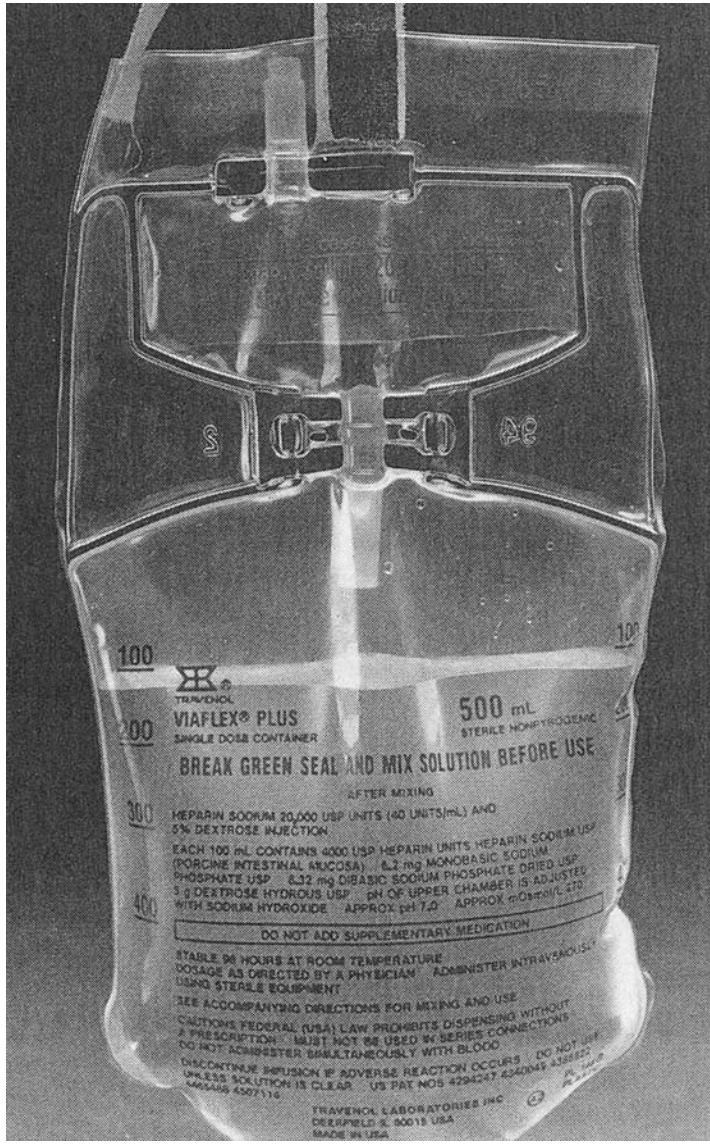


Fig. 4.24 Heparin dextrose double bag. (Courtesy of Baxter Healthcare Corp., Round Lake, IL.)

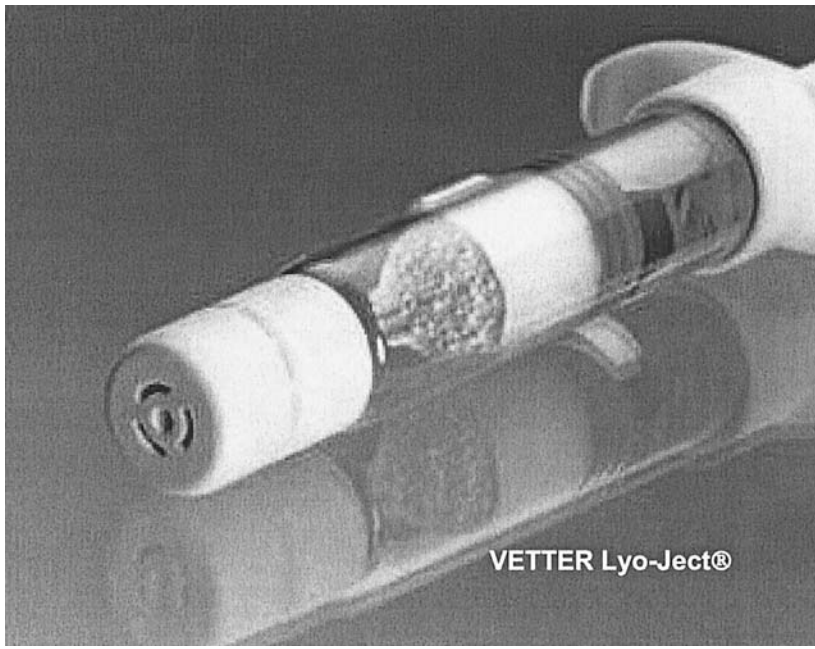


Fig. 4.25 Vetter Lyo-ject® by Vetter GmbH. (Courtesy of Vetter GmbH, Yardley, PA.)

pounds. In addition, E-beam sterilization, hydrogen peroxide gas, and pulsed light are viable sterilization options.

The ability to accurately test packages for integrity has improved with the variety of package integrity test methods available. There is a growing body of literature on parenteral package integrity that explains the concept of "critical leakage" and that provides information on the proper use of leak test methods.

Finally, today's regulatory climate requires that, for even simple package systems, the integrity of the package must be demonstrated both during development and throughout the marketed product's shelf life. This requires that package integrity be incorporated in the product development program

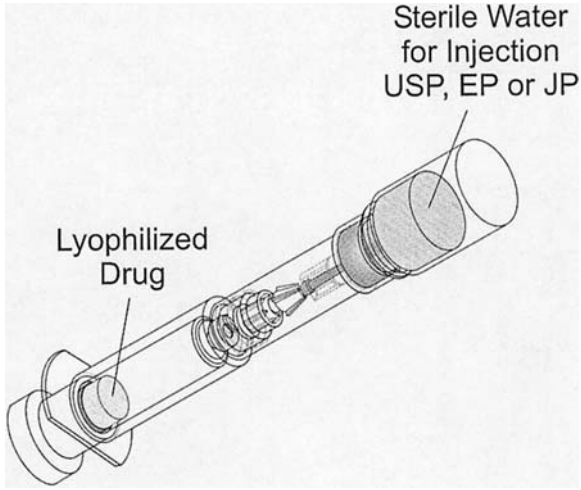


Fig. 4.26 Inter-VialPLUS System by Duoject Medical Systems, Inc. (Courtesy of Duoject Medical Systems, Inc., Bromont, Canada.)

and sustained as a component of marketed product stability studies.

PACKAGE INTEGRITY AND THE PRODUCT LIFE CYCLE

Now that the concepts of leakage have been explained, some of the package integrity test methods have been described, and the challenges of newer package designs have been touched upon, it is time to explore how package integrity fits into the product's life cycle, from early development to marketed product shelf life expiry.

Package Selection

To ensure successful product development, it is necessary to follow a carefully outlined approach to package development. Package components should be rationally selected based on their physicochemical compatibility with the product, as well

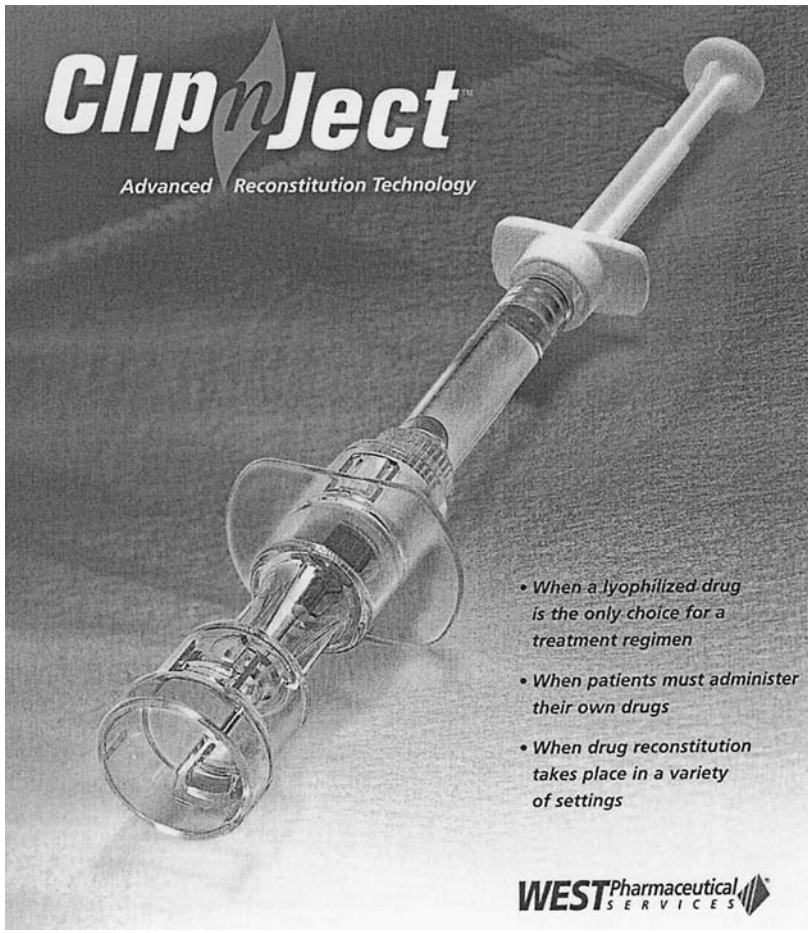


Fig. 4.27 Clip'n'Ject® by West Pharmaceutical Services, Inc. (Courtesy of West Pharmaceutical Services, Inc., Lionville, PA.)

as for their proper fit and final package functionality, and for their ability to withstand processes such as sterilization and the anticipated product distribution cycle. Critical dimensions need to be defined and the specifications set to ensure satisfactory package performance under worst-case conditions. The dimensions of the components should be specified according

Fig. 4.28 Bio-Set® Luer Injection System by Baxter Healthcare Corporation. (Courtesy of Baxter Healthcare Corp., Round Lake, IL.)

Fig. 4.29 Viringe™ Vascular Access Flush Device, Avitro, Inc. (Courtesy of Avitro, Inc., Zephyr Cove, Nevada.)

to appropriate fit, clearance, and interference. Component samples representing multiple component lots should be evaluated at the dimensional specification limits for their impact on package functionality and package integrity.

Package Function and Leakage Specifications

When designing a parenteral product package, it is important to include a definition of the package's intended functions. All parenteral packaging must maintain the sterility of the contents. In addition, the package must contain the liquid or dry powder product. This may include the maintenance of low levels of moisture or oxygen or low atmospheric pressure in the package headspace. For multicompartment packaging (for example, wet/dry or wet/wet packaging systems), it is important that the contents of each chamber be kept isolated until the time of mixing. The package should also function appropriately to deliver product to the patient. For example, a prefilled syringe must break away and extrude with reasonable force and without leakage of the contents, package/delivery system combinations must mate without contamination or loss of product, and a stoppered multidose vial must reseal after puncturing and not core.

Once these functions have been identified, leak rate specifications can be established. For example, a container intended to simply maintain sterility and prevent the loss of the liquid contents needs to meet appropriate leak rate specifications that reflect liquid leak limits. Containers required to preserve a defined headspace will need to be assigned a tighter specification based on the allowable headspace fluctuations. More than one specification may be needed to differentiate between long-term storage performance and point of use functionality. For example, a multidose lyophilized or powder-filled vial may need to meet rather strict gaseous leakage specifications during its shelf life. Once reconstituted, it is only important that no liquid leakage occur, and that any microorganisms present in the atmosphere or on the outer package

surface be prevented from entering the system. After leakage specifications have been agreed upon, finished product leak test methods can be selected to reflect these specifications. Leak test methods should optimally include physical tests designed to provide rapid, clear, and reliable results for screening packages throughout the development process.

Package Filling and Assembly

Processing and assembly variables should be understood for their potential impact on package integrity. During production, the product is filled into the package and the package is closed. This assembly process may include, for example, torquing a cap onto an ophthalmic bottle, crimping an aluminum seal onto a stoppered vial, or flame-sealing a glass ampule. Each process entails several variables that ideally should be evaluated at the proposed operational limits for their impact on package integrity. Each of these variables should be monitored by some physical or mechanical means to ensure that the assembly process is kept under control. For example, flame-sealed glass ampules need to be tested for integrity when sealed at the limits of gas and oxygen flow rate, filling speed, ampule seal neck target location, and product fill volume. Screw-cap containers need to be tested for cap removal torque and leakage over time as a function of initial capping torque force. To assure adequate package integrity, it is useful to assess components made at the dimensional specification limits and to compare multiple component lots for their dimensional and functional consistency.

Components tested should include some that have been processed and sterilized according to worst-case conditions known to cause component physical deformation or degradation. The impact of postassembly processes, such as terminal sterilization, on package integrity certainly must be evaluated. Sterilization cycle validation should include package integrity checks under the worst-case conditions of package design, assembly, and sterilization.

Finally, careless or improper handling of components and packages have resulted in package integrity failures and should be prevented through proper procedures and training.

Packaged Product Distribution

The distribution environment challenges of temperature, pressure, humidity, shock, and vibration should be considered in evaluating containers for integrity. In today's world of heightened security, the impact of X-ray exposure should also be considered. To test for the effects of the distribution environment on package integrity, packages may simply be shipped by appropriate means of transportation. Sophisticated monitoring systems are available that can record the temperature, humidity, and/or the shock/vibration environment actually seen by the product during shipment. Such tests give valuable "real-life" data; however, it is difficult or impossible to isolate the cause of a reported package integrity failure. To gain insight into specific integrity test failures, simulated shipping studies can be performed in the laboratory.

Packaged Product Storage

It cannot be assumed that package integrity of first-manufactured product remains unchanged over its shelf life. Aged product will often exhibit different package integrity over time. Frequently, this is due to the changes in inherent viscoelasticity of polymeric materials used in packages. This is exemplified by the decay in residual seal force seen with stoppered vials or the drop in removal torque of screw-capped bottles. Heat seals formed between polymeric materials may become brittle with time and adhesives may lose their bonding efficiency. Other changes in packages are the result of direct interaction of the product with the package. For instance, a product's solvent system may result in swelling or shrinkage of components and subsequent improper fit. This is the case when shrinkage of an elastomeric gasket on an aerosol container valve results in loss of propellant and/or product.

Package changes that impact integrity may also affect the package's functionality. For instance, silicone sorption into a syringe's elastomeric plunger may ultimately cause poor breakaway and extrusion performance. Aged multidose vial elastomeric closures may lose their ability to effectively reseal and exhibit increased coring tendencies, both of which are related to package integrity as well as functionality.

Therefore, package integrity is not complete until the effects of storage temperature and humidity over shelf life are monitored. Accelerated aging studies at elevated temperatures can prove valuable in predicting these effects. However, it is important to verify these results using real-time stability data. For this reason, primary stability studies used to support a regulatory submission for new product approval should include appropriate package integrity and functionality tests.

Marketed Product Shelf Life Stability

The assurance of package integrity should be determined during product development by defining the relationships between leakage rate and package materials/dimensions, processing/assembly parameters, and product stability/distribution. Once these relationships are established, the institution of proper quality assurance specifications and manufacturing controls and monitors can ensure that the components and processes are kept within allowable limits and will provide assurance of package integrity. Further leakage tests should only be necessary to verify control of the processes and the package. To satisfy regulatory demands, it may be necessary to include appropriate package integrity tests that demonstrate a package's microbial integrity as part of the marketed product stability protocol.

CONCLUSION

In conclusion, package integrity is a simple concept, but one that is not easily measured or validated. Scientifically speaking, leakage is a quantitative term mathematically described

as the amount of gas capable of passing through a seal under carefully defined conditions of temperature and pressure. In the same way that nothing is absolutely "clean" or "pure," most packages exhibit some degree of leakage. In other words, a "leak-tight" package is one with leaks so small as to be inconsequential. Verifying package integrity is therefore a matter of defining the leakage specification limits and selecting an appropriate test method(s) for detecting leakage at these limits. Leakage specifications should be conservative enough to guarantee sterility of the parenteral package and to ensure satisfactory product stability and package performance throughout the shelf life, but not so stringent as to make integrity testing verification prohibitively difficult and expensive. When choosing a leak test method, the sensitivity of the equipment or technique should be weighed against other factors, such as reliability, reproducibility, speed, cost, user friendliness, data integrity, and data-processing capabilities.

The pharmaceutical industry can no longer take a lackadaisical approach to package integrity validation. The growing demand for novel, more complex package systems has brought new challenges to package design and development. There are a variety of package materials, and there are numerous sterilization methods available to permit the creation of such unique package systems. The ability to accurately test packages for integrity has improved with the variety of package integrity test methods offered. This is coupled with a greater understanding of leakage concepts. Finally, new parenteral product approvals hinge on the data package provided to regulatory authorities that satisfactorily demonstrate the package's ability to function and remain integral over anticipated conditions of distribution and storage for the life of the product.

To meet these challenges, leaders in the pharmaceutical industry are taking a more comprehensive product/package development approach that incorporates the demonstration and validation of package integrity and functionality. This begins by careful selection of package component materials and

design. The stability, functional performance, and integrity expectations of the package need to be defined early in the development process. This should be followed by the optimization of package filling, assembly, and processing operations. Whenever possible, the integrity and functional performance of the package should be challenged at the limits of component dimensional specifications and at the operational parameter extremes of filling, assembly, and processing. Checks are to be integrated into each critical step to monitor and ensure adequate packaging and process controls. Finished product should also be evaluated as a function of the distribution and long-term storage environment. Once product development is complete, continual monitoring of critical steps in production serve as the primary tool for assuring package integrity. Additional leakage tests to demonstrate microbial integrity of the marketed product over shelf life then provide the final check for a thoroughly validated product/package system.

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Appendix I

Example of Standard Operating Procedure for Sterility Testing by Direct Inoculation*

Purpose: To describe the USP test procedure for sterility testing by direct inoculation.

Equipment and Supplies

1. Trypticase soy broth (TSB) medium—sterile test tubes of appropriate size, one for each sample plus three controls
2. Fluid thioglycollate medium (FTM)—sterile test tubes of appropriate size, one for each sample plus three controls
3. Sterile syringes or pipets, one for each sample
4. One incubator at 30–35°C
5. One incubator at 20–25°C

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6. Laminar flow (LF) workbench
7. Sterile Tyvek gown
8. Sterile disposable cap
9. Sterile mask
10. 70% Alcohol
11. Betadine or pHisohex
12. Urethane wipes

Procedure

Steps	Comments
1. The operator shall wear a sterile coat, mask, hood, and disposable cap. The operator shall wash hands thoroughly with a disinfectant scrub before donning sterile gloves.	
2. Wipe inside, top, and counter surfaces of LF workbench with suitable surface disinfectant.	Take care to avoid splashing surface of HEPA filter. Surface disinfectant is usually 70% alcohol, but others may be used and should be from time to time.
3. Wipe all exposed surfaces of vials, ampules, tubes of culture media, and other containers with surface disinfectant before placing them in LF workbench.	
4. All sterile items having an outer wrapping should have the wrapping removed at the edge of the LF workbench and the sterile inner item introduced aseptically into the workbench.	Alternatively, the outer wrapping may be wiped with a disinfectant prior to placing in the workbench, but this is less desirable.

Steps	Comments
5. After all supply items have been introduced into the LF workbench, the operator should change to a new pair of sterile gloves, or preferably, partner will then perform the critical aseptic steps using uncontaminated gloves.	The usual hand disinfectant is pHisohex or betadine. Sterile latex or PVC gloves may be worn, but are not required.
6. For vials, remove protective seal and disinfect exposed rubber closure with alcohol wipe.	Leave damp, but there should be *no* pool of disinfectant.
7. For ampules, break neck at score mark, pointing toward side of workbench rather than HEPA filter.	Avoid splattering of HEPA filter with liquids. Do not place hands between filtered air source and critical site.
8. Use a sterile syringe or pipet to transfer the appropriate volume of product to each test tube containing either sterile TSB or FTM.	Refer to USP sterility test procedures for appropriate volumes of media and product inocula.
9. After all required product samples have been inoculated, inoculate one additional tube of TSB and FTM with product. Then, inoculate each tube with a loopful of standard test organism culture of a spore strip.	These tubes will serve as positive controls to show that the test organism grows in the presence of the product.
10. Two additional tubes of TSB and FTM should be used as controls. One tube of each medium should be inoculated with a loopful of test organism and the other tube left uninoculated.	The inoculated tubes will show that the culture media support growth of microorganisms and the blank tubes will confirm the sterility of the culture media.

	Steps	Comments
11.	Incubate the samples at appropriate temperatures and observe after 3, 7, and 10 days for presence of microbial growth.	Incubate FTM at 30–35°C and TSB at 20–25°C (room temperature [RT]). Longer incubation may be required at times to permit slow growers to develop.
12.	Record results on quality control record sheet.	
13.	Sterilize used culture media and clean tubes after incubation.	

Appendix II

Example of Standard Operating Procedure for Sterility Testing by Membrane Filtration*

Purpose: To describe a method for the use of membrane filters in the sterility testing of sterile products.

Equipment and Supplies

1. Sterility test filter holder unit—Millipore Sterifil, Falcon unit
2. Membrane filter, 0.45 mm, 47 mm, hydrophobic edge
3. Sterile needles, syringes, or administration sets—20
4. Sterile trypticase soy broth (TSB), 100-ml tubes—3
5. Sterile fluid thioglycollate medium (FTM), 100-ml tubes—3

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6. Sterile diluting fluid, 100 ml—3
7. Sterile scissors
8. Sterile forceps, smooth tip, 4–6 inch stainless steel
9. Sterile disposable gloves
10. Alcohol, 70% denatured
11. Urethane wipes
12. Laminar flow hood (LFH)
13. Blue plastic base with hole for Falcon unit
14. Test samples
15. Sterile gown, cap, and mask

Procedure

Steps	Comments
1. The operator shall wear a sterile coat, mask, hood, and disposable cap. The operator shall wash hands thoroughly with a disinfectant scrub before donning sterile gloves.	
2. Wipe inside, top, and counter surfaces of LF workbench with suitable surface disinfectant.	Take care to avoid splashing surface of HEPA filter. Surface disinfectant is usually 70% alcohol, but others may be used from time to time.
3. Wipe all exposed surfaces of vials, ampules, tubes of culture media, and other containers with surface disinfectant before placing them in LF workbench.	
4. All sterile items having an outer wrapping should have the wrapping removed at the edge of the LF workbench and the sterile inner item introduced aseptically into the workbench.	Alternatively, the outer wrapping may be wiped with a disinfectant prior to placing in the workbench, but this is less desirable.

	Steps	Comments
5.	After all supply items have been introduced into LF workbench, the operator should change to a new pair of sterile gloves, or preferably, partner will then perform the critical aseptic steps using uncontaminated gloves.	The usual hand disinfectant is pHisohex or betadine. Sterile latex or PVC gloves may be worn, but are not required.
6.	Remove overseals from the necks of test samples, previously disinfected ampules, multidose vials or large-volume containers. Wipe the rubber diaphragm or neck of ampules with 70% alcohol.	Do not leave excess alcohol on closure. Allow the closures to air dry in the LF hood.
7.	For ampules, break neck at score mark, pointing toward side of workbench rather than HEPA filter.	Avoid splattering of HEPA filter with liquids. Do not place hands between filtered air source and critical site.
8.	Attach previously sterilized filter unit to vacuum sources. Filter unit should contain 47-mm, 0.45-μm hydrophobic edge membrane.	Use rubber tubing. Make certain a trap flask is used to collect filtrate overflow. Falcon units may be stabilized by setting in hold of blue plastic base.
9.	Transfer the prescribed volume from sample to upper chamber of filter unit. (a) Use a needle and syringe to withdraw the prescribed inoculum of product from ampules or vials. Insert needle through rubber closure of vials or into opened ampules and withdraw prescribed sample for test, or (b) use a needle and transfer set to transfer the prescribed volume of solution from large volume containers. Insert spike of set through rubber diaphragm.	See USP sterility test for number of samples and inoculum size. One syringe may be used for all samples since the samples will be pooled, but a new sterile needle should be used for each vial or bottle.

	Steps	Comments
10.	Wipe injection diaphragm of filter unit with 70% alcohol.	
11.	Insert needle of syringe or transfer set through previously asepticized diaphragm or administration set.	Use proper aseptic technique. Be sure critical sites are bathed directly in LF air. A closed system is essential to prevent drawing environmental contaminants into upper chamber of filter unit.
12.	Inject from syringe or apply vacuum to transfer prescribed volume of solution to be tested into upper chamber of the filter.	Avoid direct injection on membrane as it may puncture the filter. Preferably, inject down side or into liquid layer above filter.
13.	Apply vacuum to pull or prime to push solution through filter.	Pull or push all solution through filter.
14.	Repeat steps 6–10 until all units have been tested.	
15.	When all solution has been filtered, turn off vacuum and carefully remove top.	Turn off vacuum carefully to avoid reverse surge. Care must be taken to avoid accidental contamination.
16.	Aseptically pour 100 ml sterile diluting fluid down internal sides of chamber and onto the filter. Replace top, apply vacuum, and filter the fluid.	To remove residual portions of product, rinse all surfaces efficiently.
17.	Repeat step 13 two more times.	
18.	After all solution has been filtered, turn off vacuum and carefully remove top half of filter assembly.	Exercise caution to avoid contamination.

	Steps	Comments
19.	Using sterile forceps and scissors, remove membrane from holder and cut into two halves.	Hold filter and cut over a sterile surface so that the membrane will not be accidentally contaminated if it falls.
20.	Place one half of the membrane in a sterile tube of SCD, the other half in a tube of FTM, and incubate at prescribed temperatures for the specified time.	Use sterile forceps to place filter in culture media tubes. See USP for incubation times and temperatures.
21.	Include a positive and a negative control tube of each medium.	Inoculate one tube of each medium with a loopful of spore suspension or a paper strip of *B. subtilis* as a positive control, plain medium as a negative control.
22.	Incubate the samples at appropriate temperatures and observe after 3 and 7 days for presence of microbial growth.	Incubate FTM at 30–35°C and TSB at 20–25°C (room temperature [RT]). Longer incubation (e.g., 10–14 days) may be required at times to permit slow growers to develop.
23.	Thoroughly wash all equipment used. Make certain to empty and clean vacuum trap flask.	This is to remove residual product and media.
24.	Return used equipment to proper locations.	
25.	At the end of incubation period observe samples for growth and record results of tests on appropriate report forms.	
26.	Sterilize used culture media and clean tubes after incubation.	

Appendix III

Aseptic Procedures at the Laminar Flow Workbench*

Note: The laminar flow (LF) workbench with HEPA-filtered air, when functioning properly, provides a Class 100 clean environment suitable for aseptic procedures. However, the procedures utilized must take advantage of the functional features of the LF workbench in order not to compromise the achievements possible therein.

1. The LF workbench should be located in a buffer area that is clean and orderly, thereby enhancing the functional efficiency of the workbench.
2. At the beginning of each workday and each shift, and when spillage occurs, the workbench surface should be wiped thoroughly with a clean, nonlinting sponge dampened with distilled water. The entire inside of the workbench should then be wiped with another clean, nonlint-

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ing sponge dampened with a suitable disinfectant, such as 70% alcohol.

3. The blower should be operated continuously. However, should there be a long period of nonuse, the blower may be turned off and the opening covered with a plastic curtain or other shield. The blower then should be operated for at least 30 minutes, and all the internal surfaces of the hood should be cleaned thoroughly and wiped with a disinfectant before use.
4. Traffic in the area of the workbench should be minimized and controlled. The workbench should be shielded from air currents that might overcome the air curtain and carry contaminants into the work area.
5. Supplies entering the buffer area should be isolated in a remote place until they can be decontaminated by removing outer packaging. That is, outer cartons and packaging materials should not be brought near the workbench. All supply items should be examined for defects prior to being introduced into the asepetic work area.
6. Supplies to be utilized in the workbench should be decontaminated by wiping the outer surface with 70% alcohol or other suitable surface disinfectants or by removing an outer wrap at the edge of the workbench as the item is introduced into the aseptic work area.
7. If the workbench is located in a non-aseptic area, such as a hospital pharmacy, before approaching the workbench, personnel must thoroughly scrub hands and arms with a detergent followed by an appropriate skin antiseptic. Each must then don a clean cap that provides complete coverage of head hair and a clean, nonlinting, long-sleeved coat with elastic or snaps at the wrist and, preferably, a solid front panel. A face mask must be worn if there is no transparent barrier panel between the operator's face and the aseptic work area or if the operator has facial hair or an upper respiratory condition that promotes sneezing and coughing.
8. After proper introduction of supply items into the aseptic

workbench, they are to be arranged in a manner such that operations can take full advantage of the direction of laminar air flow, that is, either vertical or horizontal. Supply items within the workbench should be limited to minimize clutter of the work area and provide adequate space for critical operations. A clean pate of HEPA-filtered air must be provided directly from the filter source to the critical work site. No supplies and no movement of the personnel should interpose a nonsterile item or surface between the source of the clean air and the critical work site. Therefore, no objects should be placed horizontally behind the critical work site or above the critical work site in a vertical laminar flow workbench. Also, all work should be performed at least 6 inches within the workbench to avoid drawing contamination in from the outside.

9. All supply items should be arranged so that the work flow will provide maximum efficiency and order.
10. It should be noted that the hands are clean, but not sterile. Therefore, all procedures should be performed in a manner to minimize the risk of touch contamination. For example, the outside barrel of a syringe may be touched with the hands since it does not contact the solution, but the plunger or needle should not be touched.
11. All rubber stoppers of vials and bottles and the neck of ampules should be cleaned, preferably with 70% alcohol and a nonlinting sponge, prior to the introduction of the needle for removal or addition of drugs.
12. Avoid spraying solutions on the workbench screen and filter.
13. After every admixture, the contents of the container must be thoroughly mixed and should then be inspected for the presence of particulate matter or evidence of an incompatibility.
14. Filtration of solutions to remove particulate matter is frequently necessary, particularly when admixtures have been prepared. A small volume of solution may be

filtered by attaching an appropriate membrane filter to the end of a syringe, using the plunger to force the liquid through the filter. *Note*: To avoid rupture of the membrane, force may be applied in one direction only through the filter. When larger volumes of solutions must be filtered, this may be accomplished by means of an appropriate in-line filter and an evacuated container to draw the solution through the filter or, preferably, by means of a pressure tank of nitrogen, or other inert gas, to apply pressure to the liquid in the container to force it through the in-line filter. In the latter situation, the pressure must be maintained low enough to avoid the risk of explosion of the solution container (usually a maximum of 10–12 psig). There are at least two disadvantages of the vacuum system as compared with the pressure system: (a) any leakage draws contamination into the container and system; (b) the vacuum may be lost, thereby stopping the procedure.

15. The porosity of the appropriate membrane filter is determined by the objective of the filtration. To remove particulate matter, a 1-μm porosity filter should be satisfactory. To sterilize a solution, a 0.2-μm filter would be required.
16. The completed preparation should be provided with an appropriate tamperproof cap or closure to assure the user that the integrity of the container has been maintained until the time of use.
17. The workbench should be cleaned with a clean sponge, wet with distilled water, as often as necessary during the workday and at the close of the workday. This should be followed by wiping the area with a sponge with an appropriate disinfectant.
18. During procedures, used syringes, bottles, vials, and other supplies should be removed, but with a minimum of exit and reentry into the workbench.