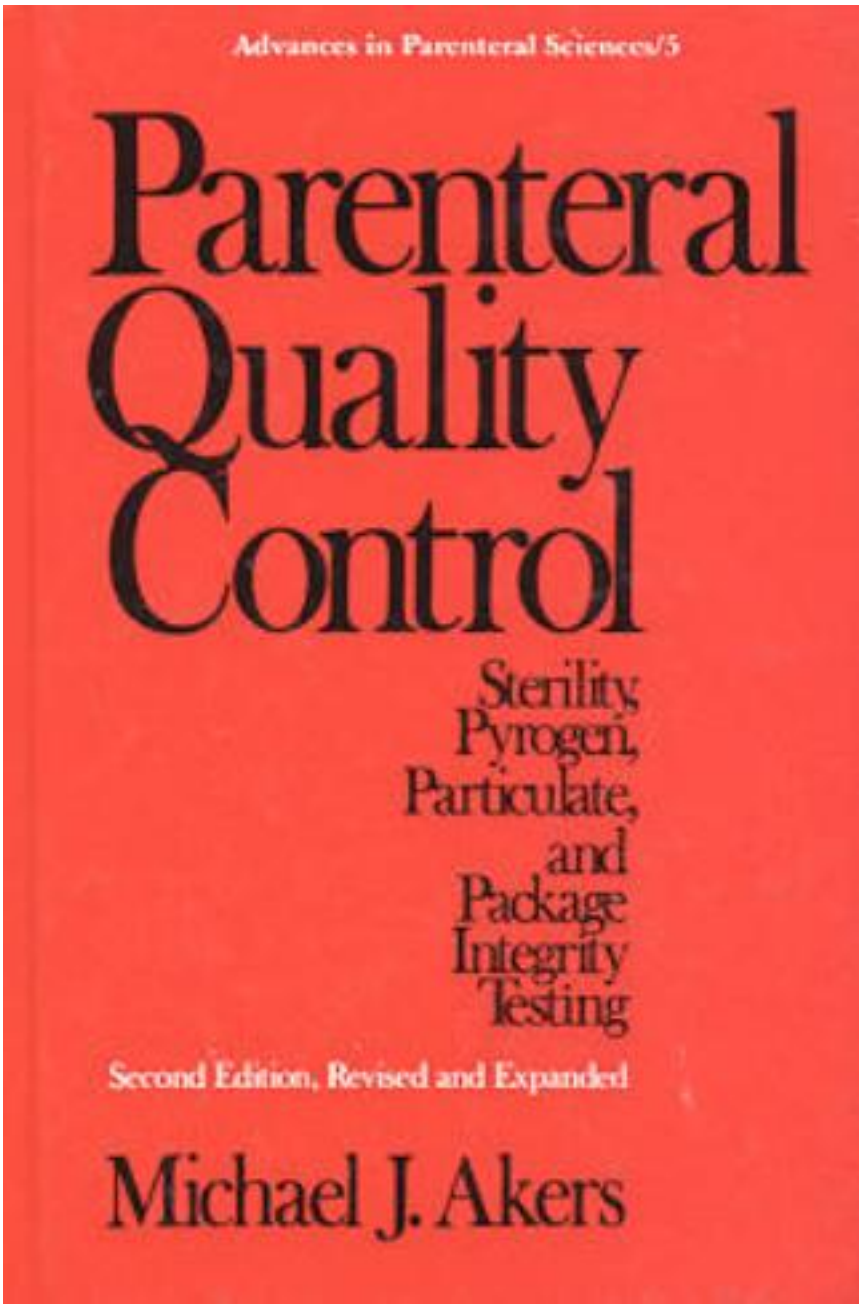

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Parenteral Quality Control

ADVANCES IN PARENTERAL SCIENCES

Editor

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Parenteral Quality Control

Sterility, Pyrogen, Particulate, and Package Integrity Testing
Second Edition, Revised and Expanded

Michael J. Akers

Eli Lilly and Company
Indianapolis, Indiana

With a Contribution by
Dana Morton Guazzo

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This book is dedicated to
Mary, Scott, Ryan, and Allison,
my beloved wife and children

Series Introduction

The field of parenterals has seen substantial technical and scientific growth over the past two decades, with the expectation of even greater activity during the last half of the 1980s and through the 1990s. This growth is due, in part, to the expected surge in the number of very potent and sensitive peptides that are arising out of the proliferating genetic engineering programs; an expected increase in the number and type of nutritional products; and the increasing demand for expanded home health care. It is entirely appropriate, therefore, that a scientific/technical series be established not only to report on advances in this field but also to help integrate the various disciplines that impact on parenteral products.

To place the series in perspective it may be useful to provide a personal impression of the level and complexity of the parenteral field. Parenteral activities can arbitrarily be divided into (1) those devoted to making an elegant, safe and effective product, (2) the interface of that product with the route of administration, and (3) the influence of the product on the time course and biological activity of the drug in question. Naturally, all three areas are related and are not easily segregated, especially the latter two.

The technical aspects of parenterals include assurance of stability, sterility, and freedom from particulates as major concerns. These areas have received considerable attention and significant advances have been made in conventional, that is, non-sustained release parenterals. Sustained forms of parenteral products require special consideration from a preparation and quality assurance point of view.

Less well studied is the interaction of the product with the biological interface, for example, biocompatibility, local metabolism and immunological reaction. These areas require a variety of disciplines to fully understand the parenteral product-biological interface. This is a field that has been studied in an uneven manner over the years, providing a less than satisfactory data base.

The last area is the influence of the product on the time course and biological activity of the drug, the so-called bioavailability issue. In the absence of a suitable understanding of the product-biological interface, that is, point number two, it is difficult to provide a thorough mechanistic description.

Thus, there is considerable need for additional work in the area of parenterals. It is hoped that the series Advances in Parenteral Sciences will provide a suitable platform to record advances in this exciting field.

JOSEPH R. ROBINSON

Preface to the Second Edition

The first edition of this book was written in 19821984. With many changes in the regulatory climate, customer quality requirements, and advances in technology, there was obviously a need to update the first edition to incorporate and highlight these changes and advances. Global testing requirements have been added to this second edition. The chapter on sterility testing contains new information on issues and approaches involving retesting of lots having failed sterility testing initially. New technologies such as barrier systems for conducting sterility testing have been added. The chapter on pyrogen testing contains new information on the *Limulus* amebocyte lysate test since this test became an official USP test for endotoxin after the first edition of this book was published. The chapter on particulate matter testing has been updated considerably with respect to small-volume parenteral testing for particulates and improvements in the detection of particulate matter in parenteral solutions. The chapter on package integrity testing has been completely rewritten through the efforts of Dr. Dana Morton Guazzo, a recognized expert in this field. Finally, all the chapters,

references, and appendices have been updated to provide information on current developments.

As with the first edition, the primary objective of this book is to provide the reader with a thoroughly referenced review of (1) past achievements, (2) current information available, and (3) both current and future issues and trends within this relatively narrow but extremely important field of parenteral quality control testing methodologies. The primary audience remains those involved or interested in the sterility, pyrogen, particulate matter, and package integrity testing of parenteral products in the pharmaceutical industry, hospitals, academia, and government. I hope that this book provides a valuable reference tool for quality testing requirements unique and specific to the parenteral product.

MICHAEL J. AKERS

Preface to the First Edition

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

Regardless of the type of dosage form, quality control testing and evaluation begin with the analysis of all raw material (drug, chemical, and packaging) specifications. During the production process, quality control is vitally involved on a daily basis in the testing, checking, and monitoring of all phases of the process. Once the final product is made, it is subjected to as many tests as are necessary to ensure the potency, purity, identity, and quality of the product. Quality control testing will

continue on the finished product throughout its shelf life to monitor the physical, chemical, and microbiological stability of the dosage form and its packaging system.

Finished product quality control of parenteral dosage forms requires additional tests corresponding to the unique properties of these dosage forms. *Sterility tests* evaluate the potential of the products to be contaminated with microbiological impurities. *Pyrogen tests* evaluate the capability of the product to cause an increase in the body temperature of rabbits or to cause a gel to form in the presence of *Limulus* amebocyte lysate. *Clarity tests* check for the presence of visible particulate contamination and *particulate analytical tests* go a step further to quantitate the number and size of particulate contamination. Additionally, for glass-sealed ampuls and other parenteral packaging systems, *package integrity tests* are performed to test the potential for the ingress of microbiological 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 the book is to critique the adequacy of current methods for sterility, pyrogen, particulate, and leaker testing and to review future trends and improved technology in these areas.

The major aim of this book is to provide an educational service to individuals in industrial, hospital, and governmental laboratories who are involved with one or more of these specific parenteral quality control testing procedures. Much of what is learned about parenteral quality control is on-the-job training and experience with little or no formal education. A single, current, and comprehensive textbook totally devoted to sterility, pyrogen, particulate, and package integrity testing, as this book claims to be, could provide a useful service and fulfill a need in the learning experience of the quality control manager, hospital pharmacist, microbiologist, and laboratory technician.

The book is divided into four chapters, in the same order as the title of the book. The approach and organization of each chapter involves

three basic concepts. First, a detailed description and analysis of the current United States Pharmacopeial testing procedure and requirements are presented. Next, what are the advantages and limitations of the current compendial testing procedures? Finally, what alternatives are available and what are future trends and methods with regard to the current testing procedures for parenteral quality control?

Major emphasis is placed on the practical application of testing methodology. A lesser, but appropriate, emphasis is given to the theoretical aspects of each type of quality control test procedure. By reading and studying this book it is hoped that the reader will better learn and understand the theory and practice of parenteral quality control.

This book could not have been completed without the significant help and advice from the following individuals: Dr. Aubrey Outschoorn, Dr. Michael J. Groves, Mr. Lowell R. Lowary, Mr. Julius Z. Knapp, and Dr. Joseph R. Robinson. I especially thank Dr. Kenneth E. Avis for providing me the opportunity to teach in these areas and develop some expertise to write a book on parenteral quality control. Many individuals were very responsive to requests for photographs, figures, and other information. These include Mr. John Connor (The West Co.), Mr. H. T. Shimizu (Eisai), Mr. Louis F. Brown (Cozzoli), Mr. Don Wright (Abbott), Dr. Robert Abshire (Alcon), Mr. C. Papastephanou (Squibb), Mr. William Lenzie (American McGaw), Dr. Henry F. Hammer (Pfizer), Mr. Richard Johnson (Travenol), Dr. Jeanne Baer (Burroughs-Wellcome), Dr. A. E. DeWald (Smith Kline & French), Dr. Alan Gray (Merck Sharp & Dohme), Mr. Paul Roman (Eli Lilly), and Mr. Tom Abbinett (Eli Lilly; deceased). Also, I thank the many manufacturers and publishers referenced in the text for their permission to use their figures and tables. Lastly, I certainly appreciate the excellent efforts of Mr. Kurt DeKemper for many of the photographs appearing in the book, Mr. Bill Kruse for all the artwork, and Mrs. Cyndi Lammers for typing the manuscript.

MICHAEL J. AKERS

Contents

1
Sterility Testing

Introduction.

Sterility, or freedom from the presence of viable microorganisms, is a strict, uncompromising requirement of an injectable dosage form. Unlike enteral administration, parenteral (Greek, *para enteron* = beside the intestine) administration of drugs avoids many of the natural protective barriers of the body. The injection of a product contaminated with living microorganisms would invite a multitude of complications to an already sick 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 United States Pharmacopeia (USP) sterility test (or other national compedial sterility test requirement). The USP sterility test provides an estimate of the probable, not actual, sterility of a lot of articles. The actual product itself administered to a patient has not been tested for sterility. The sterility test is a destructive test; thus it is impossible to test every single 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 question of the sample being representative of the whole will always be an uncertainty. 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.

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 of end-product testing to protect the consumer from being administered 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 produce sterility well beyond the capability of sterility testing to detect contamination. Release of products without end-product sterility testing but based on validation of the sterilization process is called *parametric release*.

While the sterility test does not assure sterility of every single article, it does provide the FDA as well as 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 will ensue in a later section.

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 manufacture 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 will be 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 what is 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 it is not sterile. Building sterility into a product through meticulously validated cleaning, filtration, and sterilization procedures is far preferable to the 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 one 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 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 allowed to ascertain the possibility that test failure was due to accidental contamination.

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 optimalproper nutrients, pH, temperature, atmosphere, sufficient incubation time, etc.a single microbial cell will grow by geometric progression* until the number of microbial cells and their metabolic products exceeds the solubility capability of the culture medium. Manifestation of this overgrowth is visualized by the appearance of a cloudy or turbid solution of culture media. A noxious odor may also accompany the turbid appearance of the contaminated media. The sterility test is failed by a product that generates turbidity in a vessel of culture medium while the same lot of medium without the product sample show 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 significant changes and improvements have occurred in the official sterility test requirements, a summary of which 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

*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 which replicates once every thirty minutes will, after 10 hours, grow to $2^{2\times10} = 2{,}097{,}152$ cells!

strength, quality, and purity of drugs and their dosage forms. 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 upon administration to a human being must pass the USP sterility test and 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 the 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 cGMPs was published. Sterility testing was briefly mentioned under section 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 will be covered in detail in a later section of this chapter.

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 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 200 articles, a minimum of 20 units are sampled. If the final batch size is between 20 and 200 articles then not 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

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 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 3235°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 micro-organisms

(table continued on next page)

Table 1.1 (Continued)

Year	USP edition	Change or improvement
		Section introduced on sterility testing of prefilled disposable syringes
		Provide 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 contains statement of Fo = 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

(volume > 100 ml per container), at least 10 containers are sampled. Only 10 test units are sampled for products sterilized by steam under pressure. If a product is sterilized by methods other than steam under pressure, then 20 units are sampled for sterility testing. However, if a biological indicator was used in the product batch, then only 10 test units are sampled for testing. Public Health Service and Antibiotic regulations require 20 final containers from each filling operation. Sampling requirements as specified in the USP 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 possible. 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 curves*, which are plots of probability versus percent contamination. Operating characteristic (OC) curves for sample sizes of 10 and 20 units are shown in Figures 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,

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)

		Minimum volume of each medium		
Container content (ml)	Minimum volume taken from each container for each medium	Used for direct transfer of volume taken from each container (ml)	Used for membrane or half membrane representing total volume from the appropriate number of containers (ml)	Number of containers per medium
Less than 10a	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 50a	5 ml	40	100	20
50 to less than 100a	10 ml	80	100	20
50 to less than 100, intended for intravenous administrationb	Entire contents		100	10
100 to 500a	Entire contents		100	10
Over 500a	500 ml		100	10

aIntended for multiple dose or nonintravenous use.

bIntended for single dose or intravenous use.

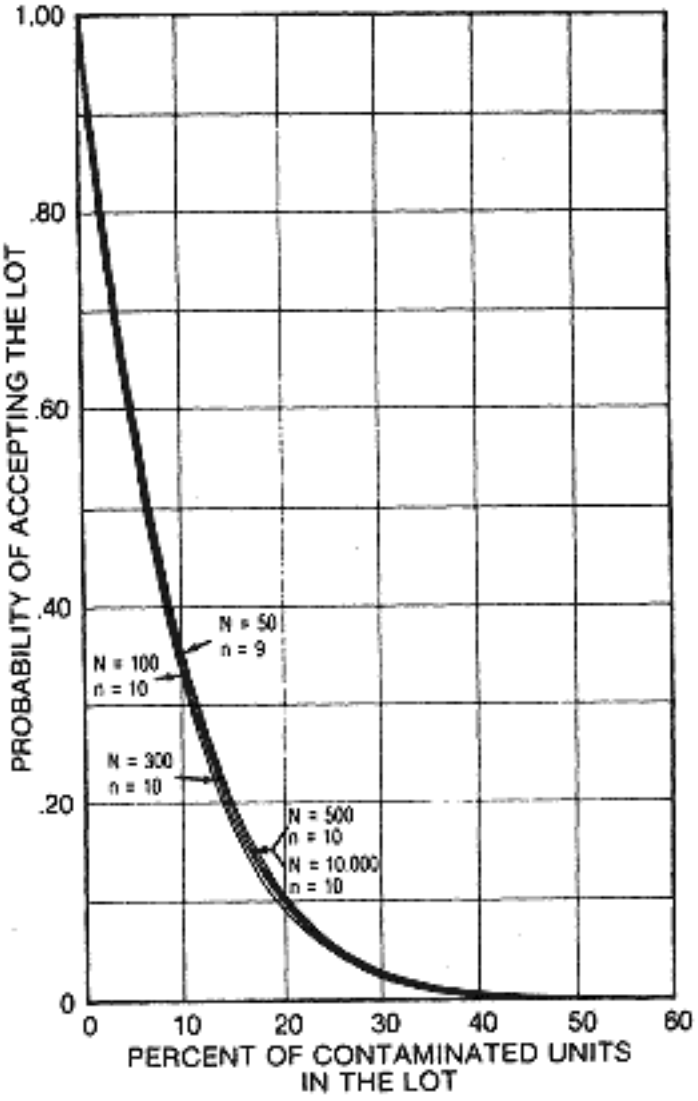

Fig. 1.1
Operating characteristic curves for a
sample size of 10 units. n = sample size;
N = lot size. (From Ref. 9.)

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 Figure 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.

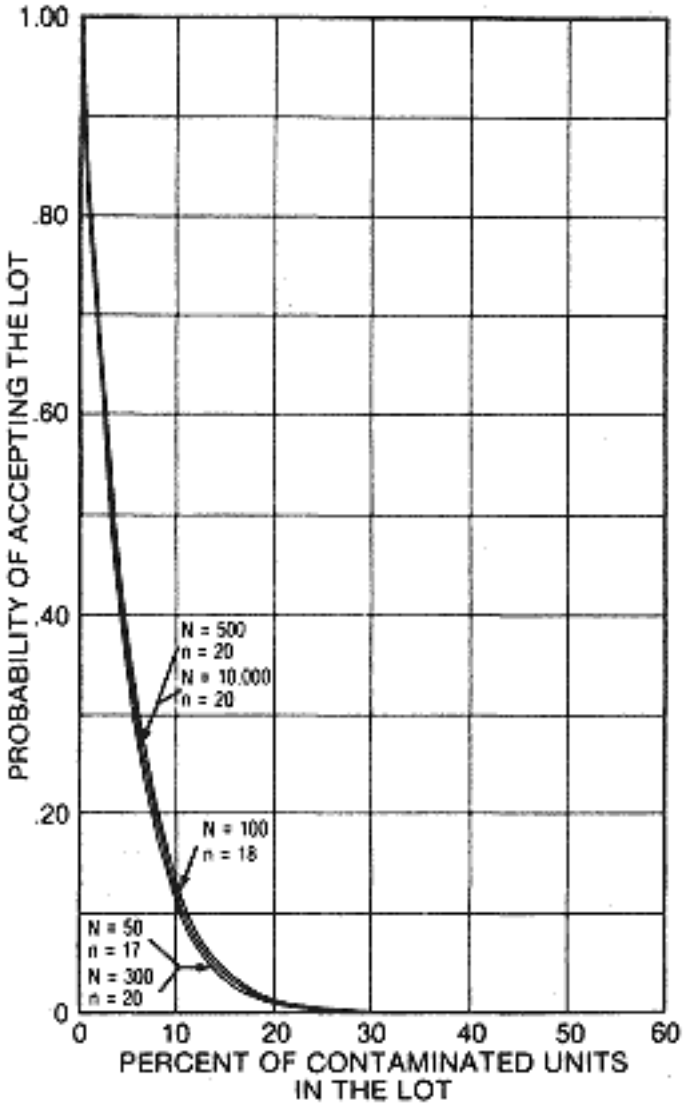

Fig. 1.2
Operating characteristic curves for a sample size of 20 units. n = sample size; N = lot size. (From Ref. 9.)

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 kth unit where k = the total units in

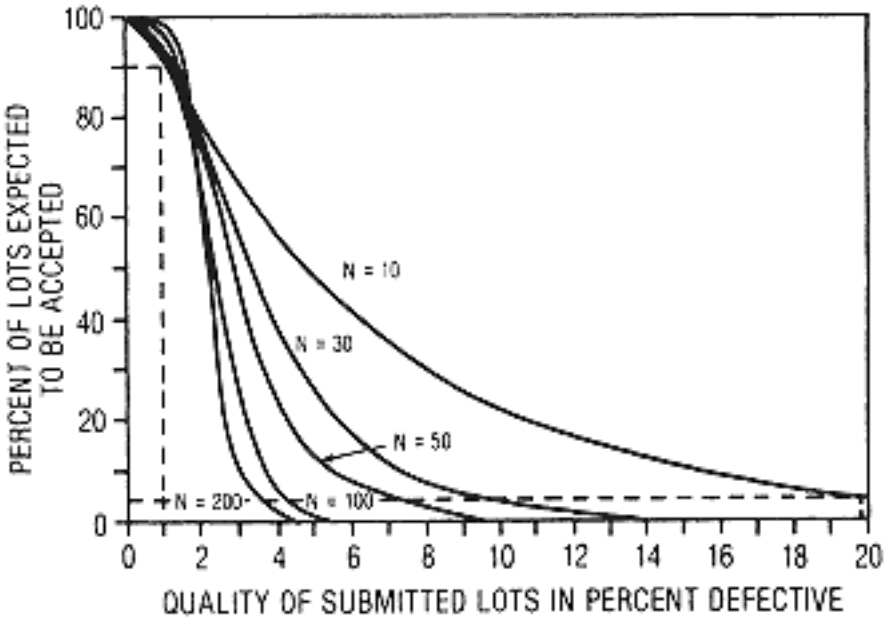

Fig. 1.3
Operating characteristic curve for sampling plans at an acceptable quality level of 1% for different sample sizes N. (From Ref. 11.)

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.*

Additional discussion of sampling with regard to its limiting the interpretation of the results of the sterility test is presented later 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 in order to prevent contamination of the sample when it is taken out of the package for testing. For example, parenteral products packaged in ampuls, vials, or bottles must be aseptically sampled using sterile materials and aseptic techniques. The neck of the ampul or the surface of the rubber closure must be disinfected with a liquid disinfectant solution before breaking the ampul or penetrating the closure with a needle. Special procedures must be implemented to

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

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 enclosures which 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 describes 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 below:

1. L-cysteine	0.5 g	Antioxidant
2. Agar, granulated (moisture content ≤ 15%)	0.75 g	Nutrient and viscosity-inducer
3. Sodium chloride	2.5 g	Isotonic agent
4. Dextrose	5.5 g	Nutrient
5. Yeast extract	5.0 g	Nutrient
6. Pancreatic digest of casein	15.0 g	Nutrient
7. Sodium thioglycollate or thioglycollic acid	0.5 0.3 ml	Antioxidant
8. Resazurin sodium solution (1:1000) freshly prepared	1.0 ml	Oxidation indicator
9. Water pH after sterilization	QS 1000 ml 7.1 ± 0.2	

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-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 surface to medium depth is very important. To provide adequate depth for oxygen penetration, a 15-ml volume of FTM must be contained in a test tube of the dimensions 20 × 150 mm. A 40-ml volume of FTM is to be contained in 25 × 200 mm test tubes, and 75100 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). *B. 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:

1. Pancreatic digest of casein	17.0 g	Nutrient
2. Papaic digest of soybean meal	3.0 g	Nutrient
3. Sodium chloride	5.0 g	Isotonic agent
4. Dibasic potassium phosphate	2.5 g	Buffer
5. Dextrose	2.5 g	Nutrient
6. Water	QS 1000 ml	
pH after sterilization	7.3 ± 0.2	

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

Other media have been proposed to replace or be substituted 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 1.3 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 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 pre-mixed culture media tubes must be obeyed, provided that the proper storage conditions (usually refrigeration) have been met.

Table 1.3 Media and Reagents Potentially Used in Performing the USP/NF Sterility Testsa

USP fluid thioglycollate (thio) medium. Use BBL 11260 or Difco 0256.

Trypticase Peptone (BBL) or Bacto-Casitone (Difco)	15.0 g
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
Distilled water	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
Distilled water	1.0 liter

Final pH 7.3

Polysorbate 80. A suitable grade is TWEEN 80, available from Atlas Chemicals Division, ICI Americas Inc.

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 continued on next page)

Table 1.3 (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
Marganous sulfate	0.3 g
Distilled water	1.0 liter
Final pH 6.5	
Saline TS, Sterile (USP)	
Sodium chloride	9.0 g
Distilled 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
Distilled water	1.0 liter

Final pH 7.3

(table continued on next page)

Table 1.3 (Continued)

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	
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
Distilled water	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	

aSterilize 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.

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 (1) weighing the appropriate amount of medium per liter of fluid desired, (2) adding water to the compounding vessel to the desired volume, (3) slowly adding the culture medium while stirring the solution, (4) applying heat and stirring until the medium is completely dissolved, and (5) 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 culture media, they must be again sterilized by steam under pressure before pouring the fluid into a drainage system and washing the containers.

When membrane filtration is used for the sterility test, a diluting fluid must be used to rinse the filtration assembly in order 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 1.4. 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 3235°C to 3035°C as required by the USP/NF (20th edition) and 3032°C specifically required by the Division of Biologics.

The current time and temperature incubation requirements of the USP sterility test are found in Table 1.5. Incubation in TSB is accomplished at 2025°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 is seven days, less than that for the direct transfer method, because of the concentrative nature of the filtration technique.

Table 1.4 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)	

The incubation time requirements of the sterility test must be of sufficient length 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 Figure 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.

Table 1.5 Time and Temperature Incubation Requirements of the USP Sterility Test

Medium	Test procedure	Time (days)a	Temperature (°C)
FTM	Direct transfer	14	3035
	Membrane filtration ≤ 100 ml	7	3035
	Membrane filtration ≥ 100 ml	7	3035
TSB	Direct transfer	14	2025
	Membrane filtration ≤ 100 ml	7	2025
	Membrane filtration ≤ 100 ml	7	2025

aTime is the minimum number of incubation days. Additional incubation time may be required if the nature of the product is conducive to produce a slow-growing contaminant.

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 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.

With 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 of the suspension to fresh culture medium between the third and seventh day after the test was started and incubate both the original and the new media for a total of, but not less than, 14 days. Thus, in all cases the original vessels would be held for 14 days. If the transfer were made on the fourth day, the transfer vessels would be held

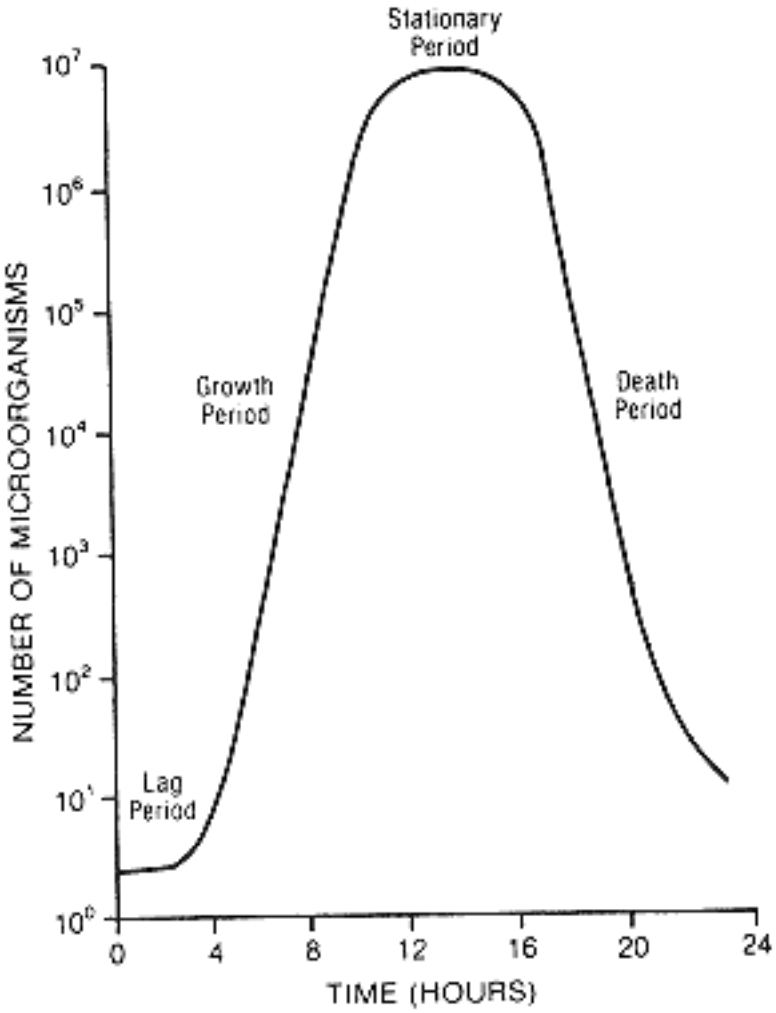

Fig. 1.4
Typical growth and death cycle for bacteria.

for 10 days. Likewise, if the transfer were made on the seventh day, the vessels would be held for seven days.

Incubation time and temperature requirements of sterility tests conducted under the auspices of various authorities are basically similar to those of the USP presented in Table 1.5. The importance of time of sampling in hospital IV admixture sterility testing was discovered by DeChant et al. (20). They found that no more than one 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 IV solutions such as dextrose 5% in water.

Sterility Test Methods

The USP specifies two basic methods for performing sterility teststhe direct transfer or direct inoculation method and the membrane filtration method, with a statement that the latter, where 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 direct transfer (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 requires a minimum volume of sample per container volume to be transferred to a minimum volume of each culture medium. Table 1.6 lists these volume requirements. The sample volume must be a sufficient representation of the entire container volume and the

Table 1.6 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 content if less than 1 ml)	15
1050	5	40
50100	10	80
100500	Total content	100
>500	500	100

volume 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 membrane filtration (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, the sterility testing of antibiotics (17), insulin (21), and large-volume parenterals (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 (Figure 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.

3. The contents are filtered with the aid of a vacuum or pressure differential system.

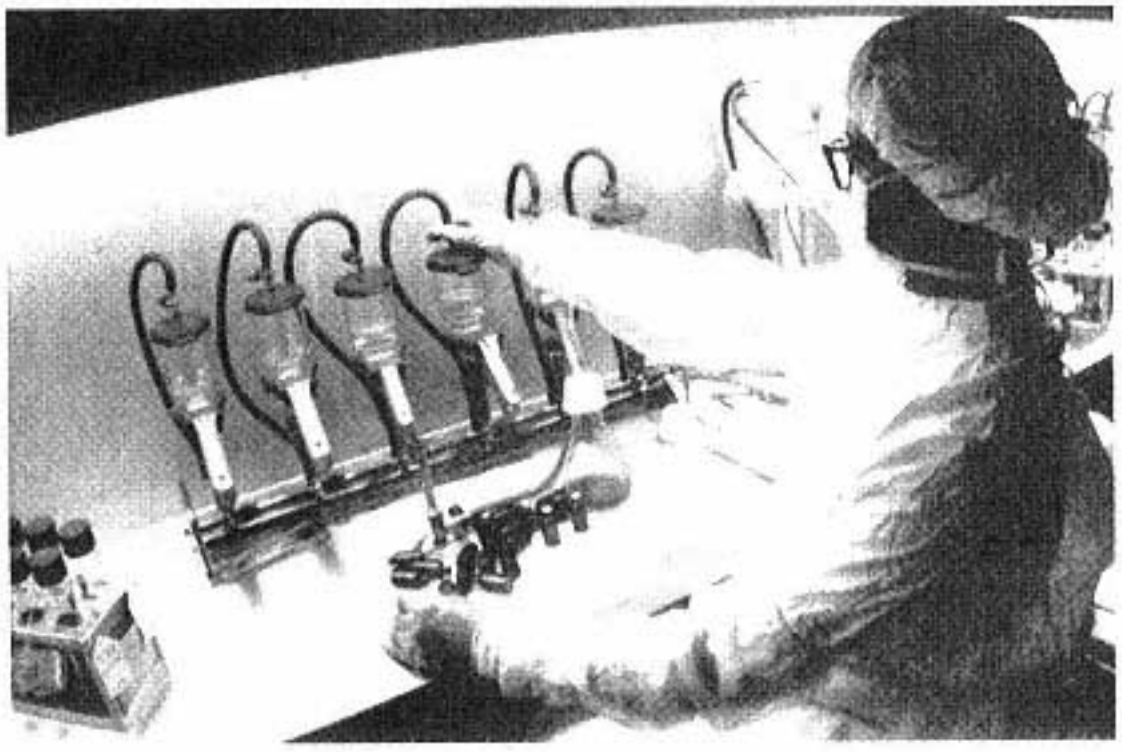

Fig. 1.5
Set-up membrane filtration sterility test apparatus. (Courtesy of Eli Lilly Co., Indianapolis, Indiana.)

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 equal volume of TSB.

The membrane used usually is a 0.450.22 micrometer porosity filter with a diameter of 47 mm and can accommodate a flow rate of 55 to 75 ml of water per minute at a pressure of 70 cm of mercury. The entire assembled unit is sterilized either by steam under pressure or by ethylene oxide. Membranes may be sterilized separately by steam under pressure or ethylene oxide; if the article to be tested is an oil, the

*The USP (22nd edition) gives the option of using two whole membranes, one for each medium, or cutting a single membrane. However, for antibiotics the CFR requires the cutting of a single membrane only.

membrane is sterilized separately in order that it may be thoroughly dried. The filter unit is then assembled under aseptic conditions.

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 MF method offers at least four advantages over the use of the DT method. They are:

1. Greater sensitivity (23).

2. The antimicrobial agent and other antimicrobial solutes in the product sample can be eliminated by filtration 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 (except when meeting requirements of Australia or Japan).

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 three major disadvantages when 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 Steritest® (Millipore) have eliminated this disadvantage.)

2. The method is unable to differentiate the extent of contamination between units, if present, because all product contents are combined

and 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

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 sterility test, may be interpreted that the sample representing the lot is absent of 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. 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 failing 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 failures 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 where a sterile product is exposed to the environment). The limitations of the USP Sterility Test (as discussed on pp. 5058) 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 that 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 a lot of consternation among 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. Table 1.7 shows a representative list 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 and, thus, QC must make a decision based on philosophical positions and retrospective history of the manufacturing and sterility test areas.

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

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, if possible, 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 historic microbial databases for the manufacturing and testing areas to assess probabilities of where the organism originated. 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

Table 1.7 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
___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 1.7 (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

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 1.7 (Continued)
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

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 whose resistance to terminal sterilization is known 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 production area. 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 = 0.5% of all sterility tests (the USP XXII allows for a false-positive frequency of not more than 2%, although the USP wishes to lower this to 1% for traditional laminar air workbench sterility test areas and 0.5% for isolation chambers used for sterility testing). Therefore, if the laboratory shows a failure rate of > 0.5% then problems must exist in either the laboratory or 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 both in the production and testing areas, can provide important information to follow up on in order 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 plates, Rotary Centrifugal Samplers, 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 issued 483 citations 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 than 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 than 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.

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. 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 Table 1.7). Any one of these records showing an aberrant result, for example, high particulate counts, lack of proof that a certain operation

was done, a sterilization run that had problems, etc., could help in determining where 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 representing a variety of departmentsQC, production, technical services, QAbefore a final decision is made regarding the acceptance or rejection of a batch of product that initially failed a sterility test.

If all review procedures fail to reveal the cause of microbial growth in the First Stage sterility test, the test will be repeated as a Second Stage test. If a cause was found to invalidate the original test, the test will be repeated as a First Stage test. A Second Stage test usually includes double the number of test samples, although the USP does not specify the particular number of specimens. A repeat First Stage test uses the same number of test samples. The minimum volumes tested from each specimen, the media, and the incubation periods remain the same as those used for the initial test.

If no microbial growth is found in the Second Stage, and the documented review of appropriate records and product details does not support the possibility of intrinsic contamination, the lot passes the requirements of the test for sterility. However, if growth is found again, the lot fails to meet the requirements of the test. The only way the Second Stage test can be repeated is for the test to be judged invalid for reasons and evidence as discussed above.

Sterility Testing of Different Sterile Products

The USP describes the sterility test procedures to be followed for all types of sterile products excluding human biologics, human antibiotics, and veterinary biologics, to which Federal regulations (9CFR113.26) apply. Test procedures for the direct transfer to test media are given for the following six types of products:

1. Liquids

2. Ointments and oils insoluble in isopropyl myristate

3. Solids

4. Purified cotton, gauze, surgical dressings, sutures, and related articles

5. Sterilized devices

6. Sterile empty or prefilled syringes

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

1. Liquids miscible with aqueous vehicles

2. Liquids immiscible with aqueous vehicles, less than 100 ml per container

3. Filterable solids

4. Ointments and oils soluble in isopropyl myristate

5. Devices

For a complete description of these test procedures, please refer to the appropriate section of the USP. In the following discussion, each test procedure will be summarized with additional information not found in the USP description for the enhancement of the reader's understanding and appreciation of the procedure.

1. Direct transfer of liquids to test media

> *Summary of procedure*: A sterile pipet or syringe and needle is used to transfer aseptically the specified volume of liquid from each test container to a vessel of culture medium. The combination is mixed and then incubated for at least 14 days. The USP describes a further procedure, previously covered in this chapter (p. 21) for transferring inoculated media when they have been rendered turbid by the test sample.
>
> *Commentary*: The risk of inadvertent contamination is at its greatest during this process. *Strict aseptic technique* must be practiced in order 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 are required to ensure that any microbial contaminants, if present, have been given sufficient time to adapt to the FTM or TSB environment and begin to thrive and reproduce.

2. Direct transfer of ointments and oils to test media

Summary of procedure: 100 mg from each of 10 containers are aseptically transferred to a flask containing 100 ml of a sterile aqueous vehicle. 10 ml of this mixture is then mixed with 80 ml of FTM and incubated for not less than 14 days. The entire process described above is repeated with another 10 containers using TSB as the medium.

Commentary: Two key facets of this procedure are: (1) employing strict aseptic technique in the *two* transfer processes for each medium, and (2) 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.

3. Direct transfer of solids to test media

Summary of procedure: The sterile solid (300 mg or the entire mass if less than 300 mg), either as is, or reconstituted as a solution or suspension with a sterile diluent, is transferred to not less than 40 ml of FTM. This is repeated for TSB. The number of containers per medium usually is 20. The incubation time is again not less than 14 days.

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.

4. Direct transfer of purified cotton, gauze, surgical dressings, sutures, and related articles to test media

Summary of procedure: The material is transferred directly to culture media using sterile instruments such as sterile forceps and sterile scissors. Sutures are contained in glass or aluminum foil enclosures. Immerse the enclosure for three hours or more in a suitable antimicrobial solution containing a dye. The enclosure is then removed with sterile forceps and viewed for evidence of leakage. If no leakage has occurred, open the enclosures aseptically and transfer the sutures to containers of FTM and TSB with sterile forceps. For petrolatum gauze, the entire contents of a single package is transferred to 300 ml of specially prepared and sterilized FTM solution at a temperature of 52°C. The mixture is shaken and cooled until the petrolatum forms a solid seal over the medium surface. The seal is broken, then the medium is incubated for not less than 7 days at 2025°C. After this initial incubation, the medium is shaken again and 0.5 ml of the mixture is transferred aseptically to 15 ml of culture medium where incubation is done again at 3035°C for not less than seven days. Media and incubation conditions are identical to those already described.

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 cut out 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. The antimicrobial solution used is either aqueous glutaraldehyde or alcoholic formaldehyde. The minimum time of three hours is necessary to assure destruction of microbial spores on the package. The dye, either crystal violet or methylene blue, is used to detect an improper or weak seal which, if

present, would allow penetration of the chemosterilizer into the container and lead to a false sterility test result (3). For petrolatum gauze, the specially prepared FTM contains more agar and the addition of gelatin to produce a more viscous medium for accommodating the petrolatum semi-solid material.

5. Direct transfer of sterilized devices to test media

Summary of procedure: The size and geometry of the device determine the type of sterility test procedure. For devices that are small enough, such as small syringes, small pipets and pipet tips, small in-line filters, needles, and the like, the entire device is immersed in not more than 1000 ml of both FTM and TSB. Fluid administration devices whose fluid pathway is claimed to be sterile are flushed with FTM and TSB, employing 20 devices per medium. The medium is emptied from the lumen, combined with additional medium (not less than 100 ml), and incubated for 14 days. In some systems having very small lumens, FTM is replaced with alternative thioglycollate medium. In the case of unwieldy devices too large to too complex in shape for the device to be sterility tested intact, the part of the article considered to be the most difficult to sterilize is removed and placed in not more than 1000 ml of culture medium. An example would be a large plastic syringe dispenser unit, such as that shown in Figure 1.6. The tubing and stopcock section is detached from the syringe and immersed in culture media. When the device or specimen has antimicrobial effects, it must be rinsed with a suitable fluid which is then tested by membrane filtration.

Commentary: A cylindrical glass culture vessel equipped with a vented nylon closure has been developed as a sterility test vessel for sterile devices of various sizes and shapes (26). The vessel is easy to charge with medium and to sterilize, has a wide opening for entry of large devices, and is less likely to allow contamination entering during storage, inoculation, and incubation.

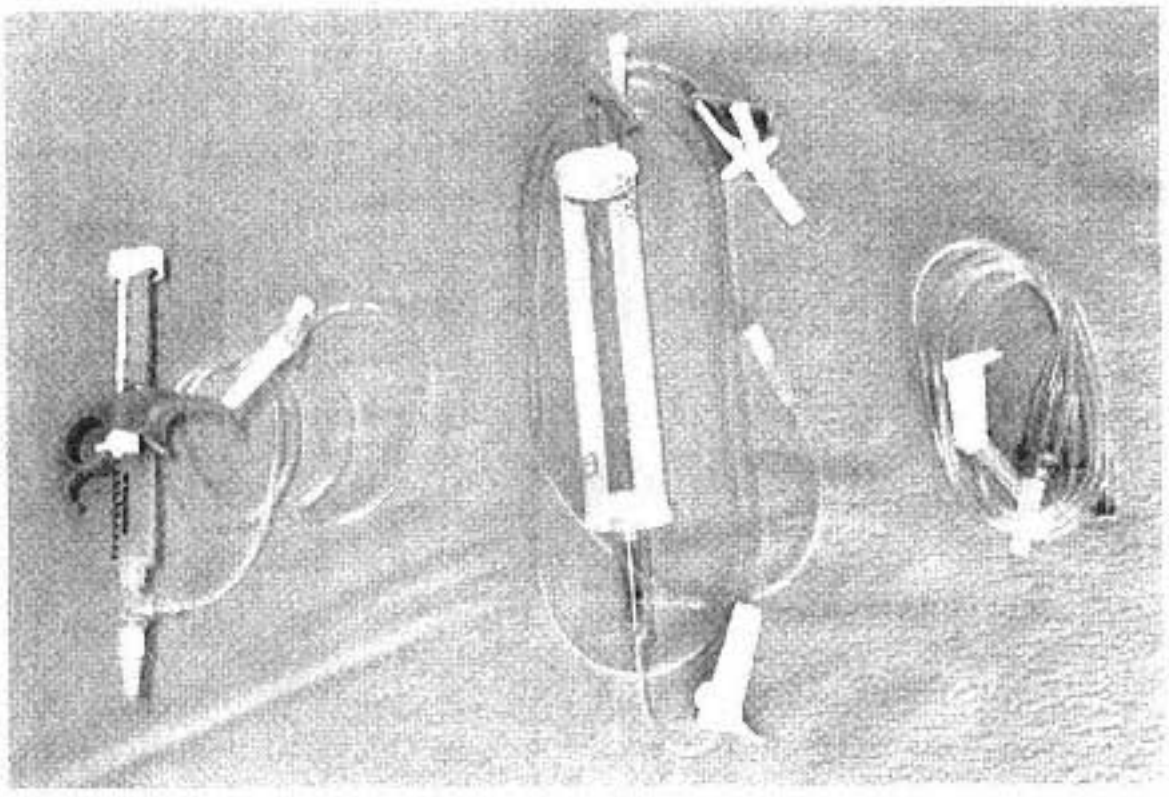

Fig. 1.6
Example of a large plastic syringe dispenser unit.

6. Direct transfer of empty or prefilled syringes to test media

Summary of procedure: The syringe product is treated like a vial or ampul in performing the sterility test. A needle is aseptically attached, if not already part of the set, to transfer the contents to vessels of culture media. After the product has been transferred, or where the syringe assembly is empty, sterile media is flushed through each syringe and/or the lumen of each needle and incubated. Each needle itself, if part of the product, is immersed in media to demonstrate sterility of the outer part of the needle.

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 where a needle must be attached later to the prefilled syringe.

The next five procedures discuss the application of the membrane filtration sterility test involving different sterile products:

1. Membrane filtration of liquids miscible with aqueous vehicles

Summary of procedure: At least 20 containers of product are used. Sufficient volumes required for both media are transferred aseptically into the membrane filter funnel. 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. Incubation is carried out for not less than 7 days.

For (a) large-volume parenteral (LVP) solutions, (b) 50 ml to less than 100 ml for I.V. 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, then 20 containers are emptied. For LVP solutions of volumes greater than 500 ml, at least 500 ml are transferred from each of 10 containers through each of two filter assemblies, 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 direct transfer. 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 have greatly reduced the number of manipulations required by the operator in using the MF technique. These systems, thus, have greatly helped to 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 upon 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.

2. Membrane filtration of 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 aseptic procedure already described and incubated in 100 ml each of FTM and TSB. For immiscible liquids of high viscosity, aseptic addition of Diluting Fluid A 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.

3. Membrane filtration of filterable solids

Summary of procedure: Six grams of dry product or a 6 g equivalent of the dry product reconstituted as a solution or suspension with a sterile diluent added to the product container is then aseptically transferred to a vessel containing 200 mL of Fluid A. If the dry product is less than 6 g per container, then take not less than 300 mg per container (or the entire contents if less than 300 mg per container) and use the same number of containers as specified for liquids miscible with aqueous vehicles.

If sample does not dissolve in 200 mL Fluid A, then use 400 mL Fluid A or divide the sample aseptically into two portions and test each in 200 mL Fluid A.

Aseptically transfer the solution(s) into one or two membrane funnels and filter. If product has inherent bacteriostatic or fungistatic properties, rinse the membranes with three 100-mL portions of Fluid A.

Commentary: The reconstitution step obviously requires strict aseptic technique. Sterile powder should never be sampled from the container in the dry state.

4. Membrane filtration of ointments and oils soluble in isopropyl myristate

Summary of procedure: Approximately 100 mg of sample from each of 20 containers is aseptically transferred to a flask containing 100 ml of sterile isopropyl myristate with a pH of water extract not

less than 6.5 that has been warmed to 44°C. The mixture is passed immediately through one or two membrane filter assemblies. After filtration, each membrane is washed with several 100200 ml portions of Diluting Fluid D, then washed with 100 ml of Diluting Fluid A (see Table 1.4). The membranes are removed aseptically and immersed in FTM and TSB culture media containing Polysorbate 80. Test samples containing petrolatum are rinsed with medium K.

Commentary: 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).

5. Membrane filtration of devices

Summary of procedure: Diluting Fluid D is aseptically passed through each of 20 devices and at least 100 ml is collected from each device. The fluid is then filtered through a membrane filter, the membrane removed aseptically, cut in half, and each half placed in 100 ml FTM and 100 TSB, respectively.

Commentary: Several problems exist in the sterility testing of devices (31). Many devices are complex and cannot be manipulated easily. These would include prosthetic materials, large syringes, dispensing aids, and multiple-unit items. In such cases, direct transfer sterility testing may be the only practical method available. Devices must be handled in such a way so as to avoid accidental contamination. Correct aseptic technique becomes critical when sterility testing devices. The complexity of certain devices also affords greater probability of inadequate sterilization within tortuous or hard-to-reach sections of the device. Also, devices may be prepared in small lots of only a few units. Therefore, it is suggested

that those concerned with the sterilization of devices periodically monitor their established sterilization cycles by means of long-term incubation of sterility test samples to assure the absence of viable microorganisms.

Sterility Testing of Antibiotics And Proteins

Antibiotics and Antimicrobial-Containing Products

The membrane filtration (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 filtration. 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 membrane filtration 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 one 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 and every 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 will be discussed in a separate section. The types of control of sterility testing to be discussed in this section include the following: (1) positive control of the culture media, that is, the testing of the growth-promoting quality of each lot of media, (2) negative control of the culture media, that is, testing the sterility of the media, (3) 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 (4) 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 microorganisms actually been present. The USP growth promotion test is 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 Table 1.8. Growth of these microorganisms must occur in the appropriate medium within seven days' 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

Table 1.8 Test Microorganisms Required by the USP for Use in the Growth Promotion Test for Each Lot of Media Used in Sterility Testinga

Medium	Test microorganismsb	Incubation temperature (°C)	Condition
Fluid thioglycollate	*Bacillus subtilis*	3035	Aerobic
	(ATCC No. 6633)c		
	Candida albicans	3035	Aerobic
	(ATCC No. 10231)		
	Bacteroides vulgatus	3035	Aerobic
	(ATCC No. 8482)d		
Alternative thioglycollate	*Bacteroides vulgatus*	3035	Anaerobic
	(ATCC No. 8482)d		
Soybean-casein digest	*Bacillus subtilis*	2025	Aerobic
	(ATCC No. 6633)c		
	Candida albicans	2025	Aerobic
	(ATCC No. 10231)		

aATCC cultures represent reference species and their use for compendial test is predicated on their 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 not more than five passages removed from the reference species.

bAvailable from the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852.

cIf a spore-forming organism is not desired, use *Micrococcus luteus* (ATCC No. 9341) at the incubation temperatures indicated in the table.

dIf a spore-forming organism is desired, use *Clostridium sporogenes* (ATCC No. 11437) at the incubation temperature indicated in the table.

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, either the medium was not sterilized properly, contamination was introduced accidently 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 sterility by the direct transfer 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 1.8 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 membrane filtration test should be applied for those materials found to be bacteriostatic or fungistatic. Where membrane filtration 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 1.9.

Table 1.9 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 Aa

aA. 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).

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.

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 ($\geq$ 100 CFU per milliliter of product) of various microorganisms to the product and then demonstrating recovery of the organisms using the sterility test methodology. Table 1.8 provides the test organisms required by USP XXII.

Limitations of the USP/NF Referee Sterility Test

The USP referee sterility test suffers from at least three limitations: (1) the invariant uncertainty that the small sample used in the test reliably represents the whole lot, (2) the inability of the culture media and incubation conditions to promote the growth of any and all potential microbial contaminants, and (3) 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 sterilization and

sterility testing of parenteral products will alleviate many of the problems of sterility testing: (1) knowledge and understanding of the sterilization process, (2) avoidance of unfavorable environmental conditions during manufacture and testing, and (3) education of personnel in the procedures of sterility testing.

The Problem of Sampling and Statistical Representation

The probability of accepting lots having a given percent contamination is related to the sterility test sample size rather than to batch size (34). For example, if a batch is 0.1% contaminated (one nonsterile unit in 1000 units) and 10 units are sampled for a sterility test, the probability of finding one of those 10 samples to be the one 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 1.10. 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, if the contamination rate increases with batch size, the probability of acceptance decreases for the same sample size.

Table 1.10 Probability of Accepting a Batch as Sterile Assuming the Contamination Rate to be Constant at 0.1%

	Batch size		
Sterility test sample 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 lots of varying degrees of contamination to sample size is given in Table 1.11 (35). Three details may be learned assuming the data in Table 1.11 to be real: (1) as the sample size is increased, the probability of accepting the lot as sterile is decreased; (2) at low levels of contamination, for example, 0.1%, the odds of ever finding that one 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); and (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) in 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 1.12. As an example, if it is assumed that only one unit in a batch of 100,000 units is contaminated (0.001%), the probability that the one contaminated unit is among the 20 sterility test samples taken at random is 0.0002, or 2 times in one million sterility tests. Table 1.13 presents an elaborative example

Table 1.11 Relationship of Probabilities of Accepting Lots of Varying Assumed Degrees of Contamination to Sample Size

	Probability of accepting the lot (true percent contamination of lot)					
Number of samples tested (n)	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 1.12 Probability of Finding at Least One Nonsterile Unit in a Sample Size of 20 Subjected to a Sterility Test

Assumed percent nonsterile units in the lot	Probability of finding at least one 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

of why dependence on sampling and sterility is, in fact, a futile attempt to prove the sterility of a lot.

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 ml and v is the volume in ml of the test sample. For example, if 10 microorganisms are present per 100 ml and the test sample is 100 ml (20 containers × 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 following section, Support

Table 1.13 Futility of Depending on Sampling and Sterility Tests for Sterility Assurance of a Lota

	Probability of finding all negatives in samples of different sizes for various levels of contamination		
n	p = 0.1	p = 0.01	p = 0.001
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

aIf 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.

If, where there is contamination, an acceptable level of acceptance is 1 in 100 lots tested, the probability where 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; 0.001% contamination 4,500 samples; 0.0001% contamination 45,000 samples. This illustrates the futility of attempting to determine sterility levels (where 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 two inoculated tubes showed growth, the proportion contaminated may be 2/200. However, if on the occasions the positive result 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 in this chapter) makes even that possibility likely to yield an unreliable result.

Techniques and Procedures for Sterility Assurance). In fact, with low levels of non-sterile units in a lot, any reasonable sampling plan would not provide realistic results (see Table 1.13). 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 *Clostridium 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 2025°C to permit the adequate growth of facultative organisms such as enterobacteria (*Escherichia coli, Salmonella, Shigella, Proteus, Serratia marcescens*, and *Flavobacterium*) and many yeasts. FTM is incubated at 3035°C to detect mesophilic bacteria. These sterility media, therefore, are not incubated at temperatures conducive to the growth of psychophiles (predominantly pseudomads) and thermophiles (predominately 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 being a result of non-sterile 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 test is reported to range from 0.2% to 3% (1,2,41). In a poll conducted by the author of this book, 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 1.14.

Table 1.14 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 positive 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	*Penicillin*
		Aspergillus
Yeast	Air, dirt	*Candida*
		Rhodotorula
		Saccharomyces

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 (non-sterile membrane filter 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 this chapter's section Laminar Air Flow). For example, Parisi and 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 a 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 a 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 (4651).* Several methods have been suggested for evaluating sterility of intravenous admixtures (5256). 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 large-volume parenterals 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: (1) Hoffmann-La Roche, the first documented robotic system in the pharmaceutical industry (Zlotnick and Franklin (58)); (2) Farmitalia; (3) Takeda; and (4) 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 which contribute or cause the inadvertent contamination problems causing false-positive sterility tests. Each of the robotic systems described in d'Arbeloff et al. (57) was successful in eliminating adventitious contamination. For example, the Roche system had not had a confirmed false positive in over four years of operation.

*This has been more recently substantiated by the Draft Guidelines on Quality Assurance for Pharmacy-Prepared Sterile Products (*Am. J. Hosp. Pharm., 49*, 407417, 1992) which propose that pharmacists establish quality assurance procedures, including sterility tests, for pharmacies which are involved in the preparation of sterile products.

Major disadvantages of the robotic sterility test systems are their expense in installation and maintenance, slower speed in conducting sterility tests, and increased complexity in 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 airflow 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 its corrosive problems. Acceptable disinfectants of 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, for example, worst case operator contamination in introducing vials into the system, in order to show that the design of the system, its optimized laminar airflow, and the removal of any further human intervention indeed remove seeded microbial contamination on product containers.

The LaCalhene isolation chamber is the most widely used sterility test chamber system in the parenteral industry today. The LaCalhene module (Figure 1.7) is made of polyvinyl chloride supported externally by a framework of stainless-steel rods. The barriers can be accessed by the operator either through glove sleeves or half suits. Materials can be introduced or removed from these barriers through a double door transport port sterile transfer door. Room air enters and exits through a 0.3-micrometer HEPA filter. LaCalhene offers many different types of isolation chambers in terms of design and function. All sterility test operationsproduct container surface decontamination, sterility test manipulations, and incubation of samplesall occur within the barrier system.

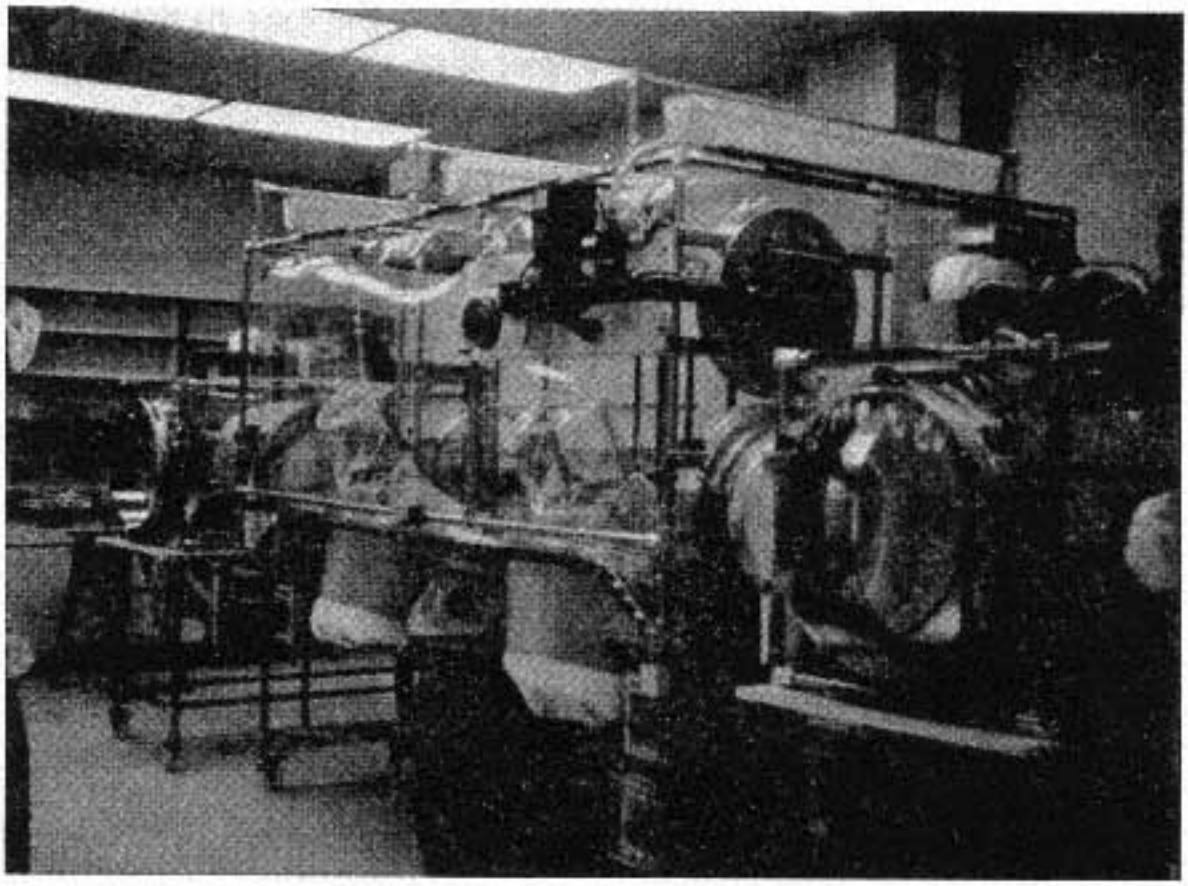

Fig. 1.7
Example of a LaCalhene isolation barrier system for sterility testing (courtesy of Eli Lilly and Co., Indianapolis, Indiana).

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 usual method of surface sterilization is 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*).

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 0.1% 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 where companies who have implemented isolation chambers or sterility test robotics will be expected to maintain lower levels of false positives than companies who 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.

Support Techniques and Procedures For Sterility Assurance

Because sterilization 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. Additionally, as has already been 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: (1) sterilizer and sterilization method validation using physical and biological indicators, (2) impeccable control of the environment conditions under which the parenteral product is manufactured particularly where aseptic processing is performed, and (3) thorough training of personnel of the strict aseptic techniques required for performing the sterility test. Any sterility test should be done in an environment no less controlled than that used for aseptic processing. An important part of long-range sterility assurance is adequate documentation of the validation, monitoring, and batch manufacturing procedures used.

Sterilizer and Sterilization Method Validation

The assurance of parenteral product sterility primarily depends upon 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. They are:

1. Heat, both wet (steam) and dry heat

2. Gas, primarily ethylene oxide

3. Radiation, primarily cobalt 60 gamma irradiation

4. Filtration through bacterial retentive membrane filters

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 expressions 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 biological indicators (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 106 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 biological indicators for the various sterilization processes are given in Table 1.15. 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 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 niger* (66). Vegetative cells, rather than bacterial spores, are employed in testing and validating filtration sensitization. *Pseudomonas 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.

The USP provides a general description of BIs. BIs are available either as liquid suspensions or as dried preparations on carriers such as paper strips, glass, or plastic beads. 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.

Table 1.15 Performance Characteristics of Biological Indicators on Paper Strips

Culture	Sterilization process	D value	Approx. 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	1.31.9 min	106	3.9 min	19 min
Bacillus subtilis subsp. *niger* (ATCC 9372)	Ethylene oxide at 54 ± 2° and relative humidity 60 ± 10%: 600 ± 30 mg/liter	2.65.8 min	106	7.8 min	58 min
	Dry heat at 160 ± 5°C	1.31.9 min	106	3.9 min	19 min
Bacillus pumilus (ATCC 27142)	Ionizing radiation				
	Wet preps.	0.160.24 Mrad	106	0.6 Mrad	2 Mrad
	Dry preps.	0.120.18 Mrad	106	0.45 Mrad	1.5 Mrad
General requirement		D ± 20%	106	3 D	10 D

Several interesting review articles have been written on the principles and applications of biological indicators. Borick and Borick (3) were the first authors to write a lengthy discussion on the use of biological indicators 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 are 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

Whenever possible, sterility tests should be performed in a test area that conforms to Class 100 conditions as described by Federal Standard Number 209D (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

*Federal Standard 209E will probably be in effect by the end of 1993 with several new definitions for air cleanliness.

Table 1.16 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

Source: Courtesy of Liberty Industries, East Berlin, Connecticut.

(table continued on next page)

Table 1.16 (Continued)

Max. number of particles per cubic ft. 0.5 µm and larger	Class	Max. number of particles per cubic ft. 5.0 µm and larger
100	100	0
1,000	1,000	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: Courtesy of Liberty Industries, East Berlin, Connecticut.

comparison of the classes of air cleanliness is provided in Table 1.16. However, so far, these classes refer to levels of particulate matter, not viable microorganisms.

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, work-benches, 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 upon the removal of dioctylphthalate particles of size 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 airflow are shown in Figures 1.8 and 1.9, respectively. Room air is sucked into

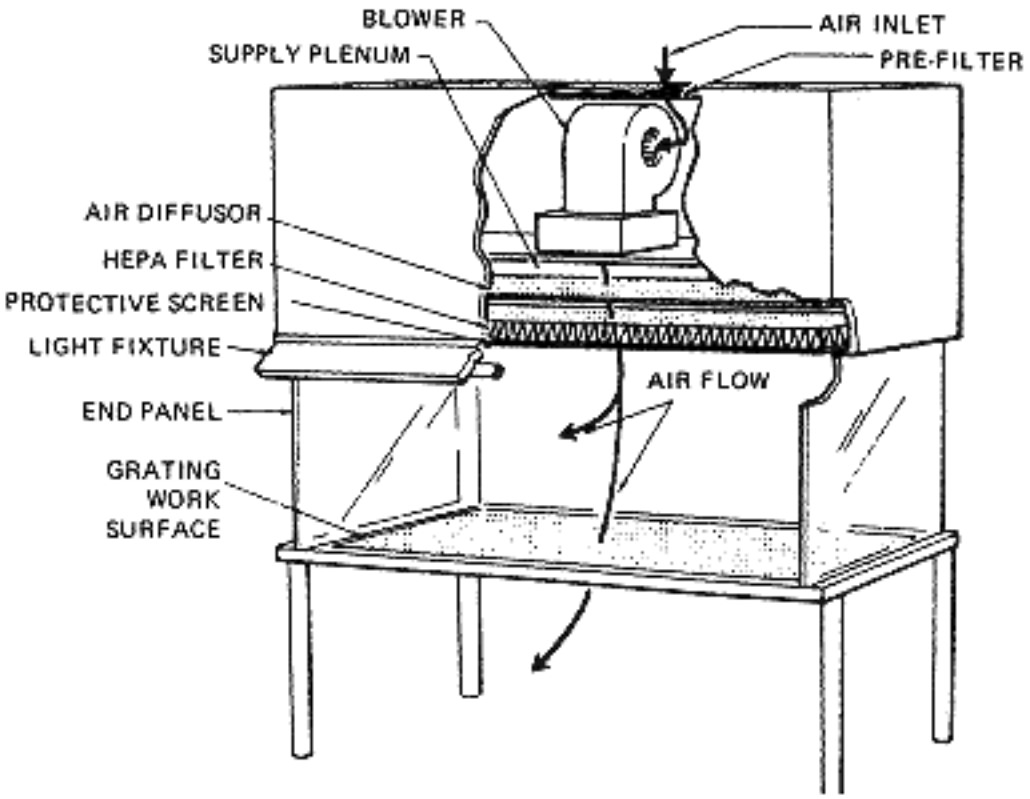

Fig. 1.8
Vertical laminar air flow bench (courtesy of Liberty Industries, East Berlin, Connecticut).

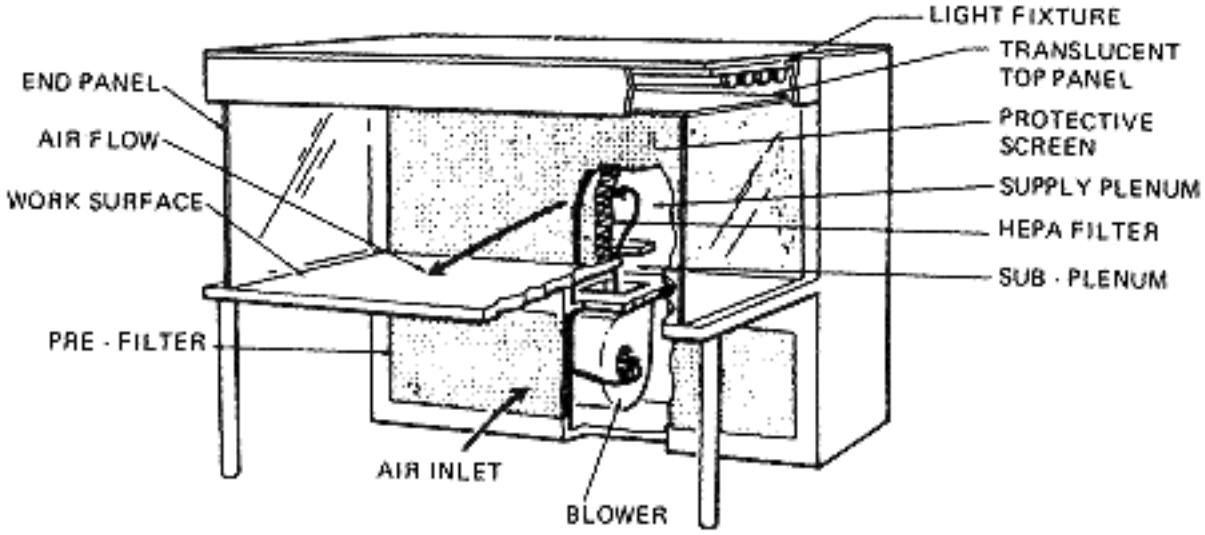

Fig. 1.9
Horizontal laminar air flow bench (courtesy of Liberty Industries, East Berlin, Connecticut).

the equipment and passes through a prefilter which removes large-sized 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 (Figure 1.10). 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 to be practiced while working at the laminar flow workbench during sterility testing are listed in Appendix IV (74).

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 periodic intervals, usually every 6 to 12 months. The velocity of HEPA-filtered air is measured using an air velometer. Air velocity at all parts of the filter should be 90 ± 20 feet per minute. Air

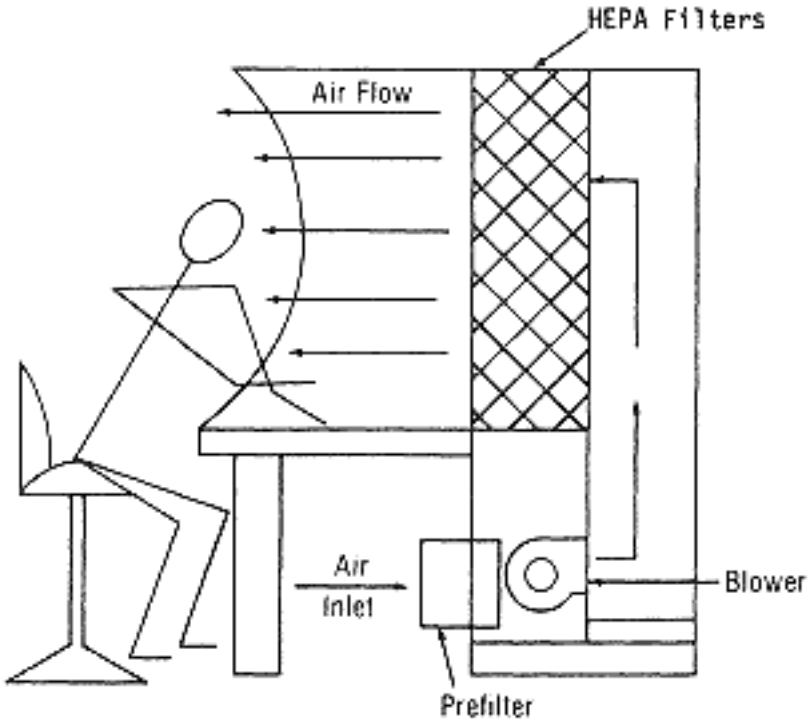

Fig. 1.10
Direction of air flow in a laminar air
flow work station (courtesy of Liberty Industries,
East Berlin, Connecticut).

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, which, 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 describing the testing of laminar flow equipment (7577). Phillips and Runkle (78) have published a comprehensive review of the biomedical applications of laminar air flow.

For most sterility testing operations, horizontal laminar air flow units appear to be superior to vertical flow hoods because the air movement is less likely to wash organisms from the operators' 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 laminar air flow workbench. For example, all glassware, containers, and other articles whose surfaces 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, etc., may be introduced into the LAF unit by removing the outer protective package at the edge of the workbench before placing the sterile item on the workbench surface. Understanding of the laminar air flow pattern is very important in order to avoid turbulence and blockage of HEPA filtered air reaching the critical work site.

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 what considerations 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 in which product and personnel move to increasingly clean environments. An example of a process flow diagram is found in Figure 1.11 (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

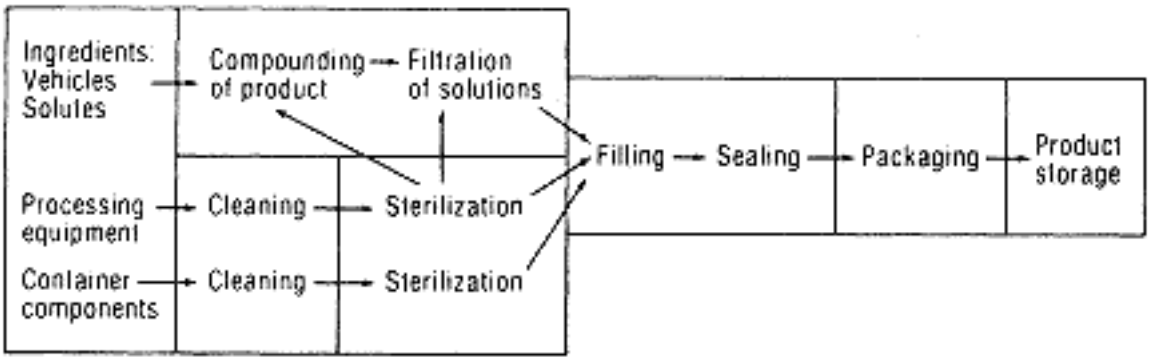

Fig. 1.11
Diagram of flow of materials through a production department (from Ref. 79).

following considerations list 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 for personnel garment storage

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 following 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 under which sterility tests are conducted. These methods can be further divided into air and surface sampling methods.

Air sampling methods

1. Slit-air sampler: This is a device that collects viable airborne microbial and particulate contamination (see Figure 1.12). 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 plate rotation and the volume of air sampled can be adjusted to record the desired rate and degree of contamination of the air environment. The slit-air sampler is one

Fig. 1.12
Slit-to-agar biological air sample (courtesy of New Brunswick Scientific Co., Inc., Edison, New Jersey).

of the most widely used monitoring methods for sterile manufacturing and quality control environments.

2. 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 microbial 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 upon 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.

3. 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.

4. Settling plates: These represent the simplest means of evaluating the microbiological quality of air. A 100 × 20 mm petri dish containing trypticase soy agar or other suitable medium is placed in a sampling location with the lid removed and placed as shown in Figure 1.13. The time period of sampling is controlledusually 3060 minutesbefore the lid is placed over the medium and the plate incubated, usually at 32°C for 48 hours. Colonies

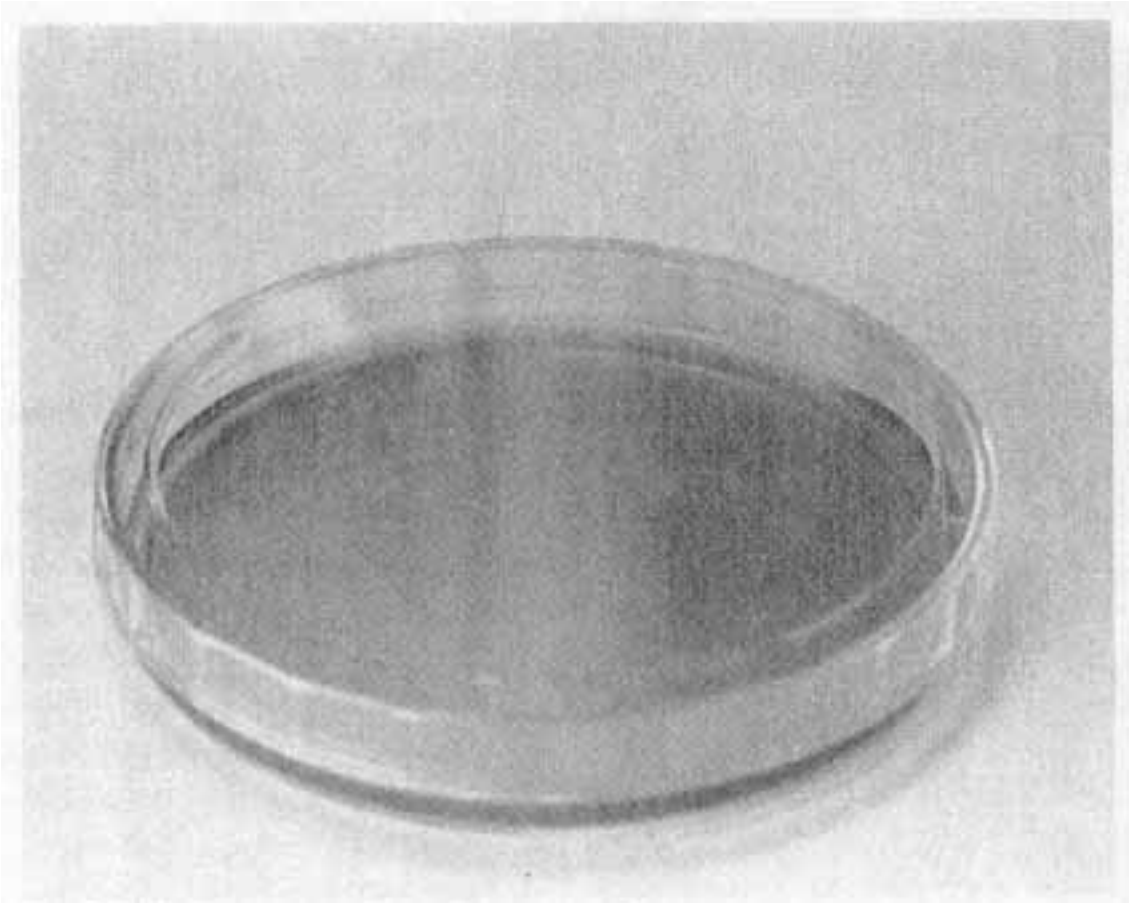

Fig. 1.13
Agar settling plate.

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.

5. Centrifugal air sampler: This device (see Figure 1.14) is the newest of the methods used to determine airborne contamination.

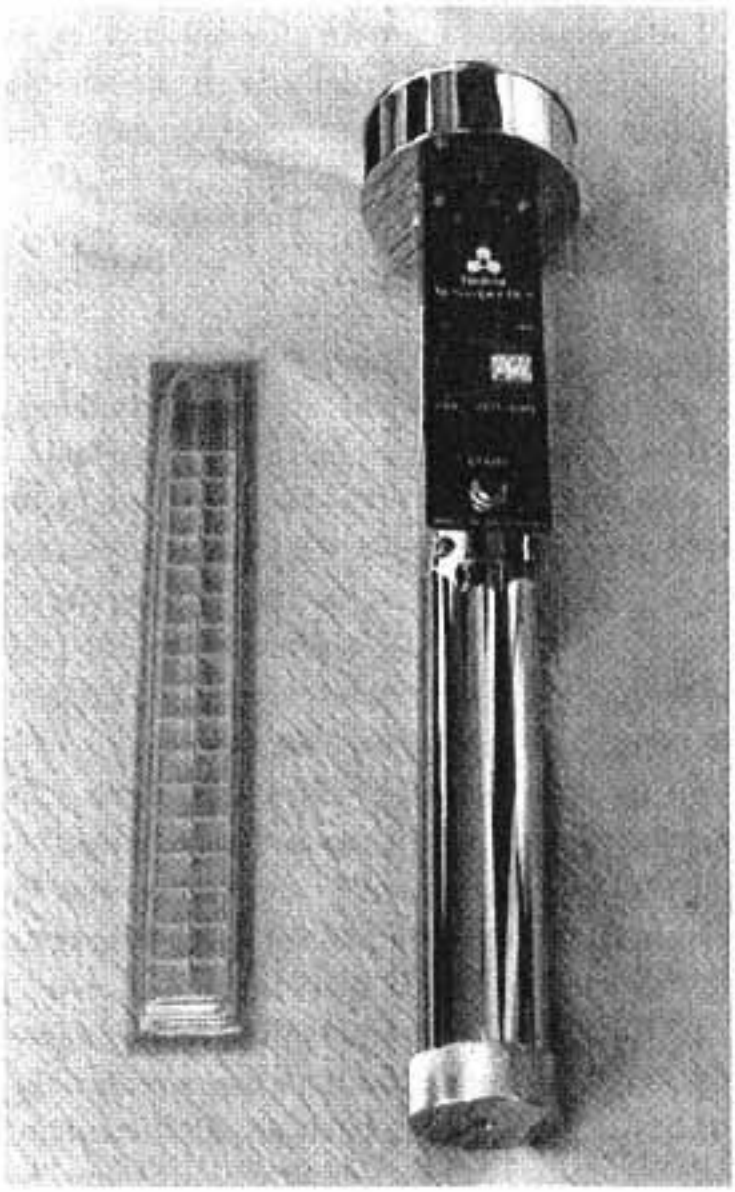

Fig. 1.14
Biotest RCS centrifugal air sampler.

Airborne microbes approximately 16 inches above the sterile drum housing are drawn toward the impeller blades. Then, owing to the applied centrifugal force, 40144178 rpm, the microbial particles are impacted at high velocity onto the agar surface of the agar strip wound around the impeller blades. After incubation colonies on the strips are counted and the results reported as colony-forming units per unit volume of air. The air capacity 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).

Surface sampling methods

1. 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 Figure 1.15). Once the molten agar has solidified, the agar surface can be gently pressed against a selected surface, for example, the surface of a laminar airflow workbench. The lid is replaced and the plate incubated for the required length of time at a controlled temperature (commonly 48 hours at 32°C). Surface contamination can be quantified by counting the colonies after incubation. The presence of Polysorbate 80 and lecithin serves two purposes, one to aid in the complete contact and removal of microbes from the sampled surface and the other to permit cleaning of the sampled area with water and/or a disinfectant solution.

2. Swab-rinse test: This is a simple surface sample 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 a sample of the water placed on a solid agar plate.

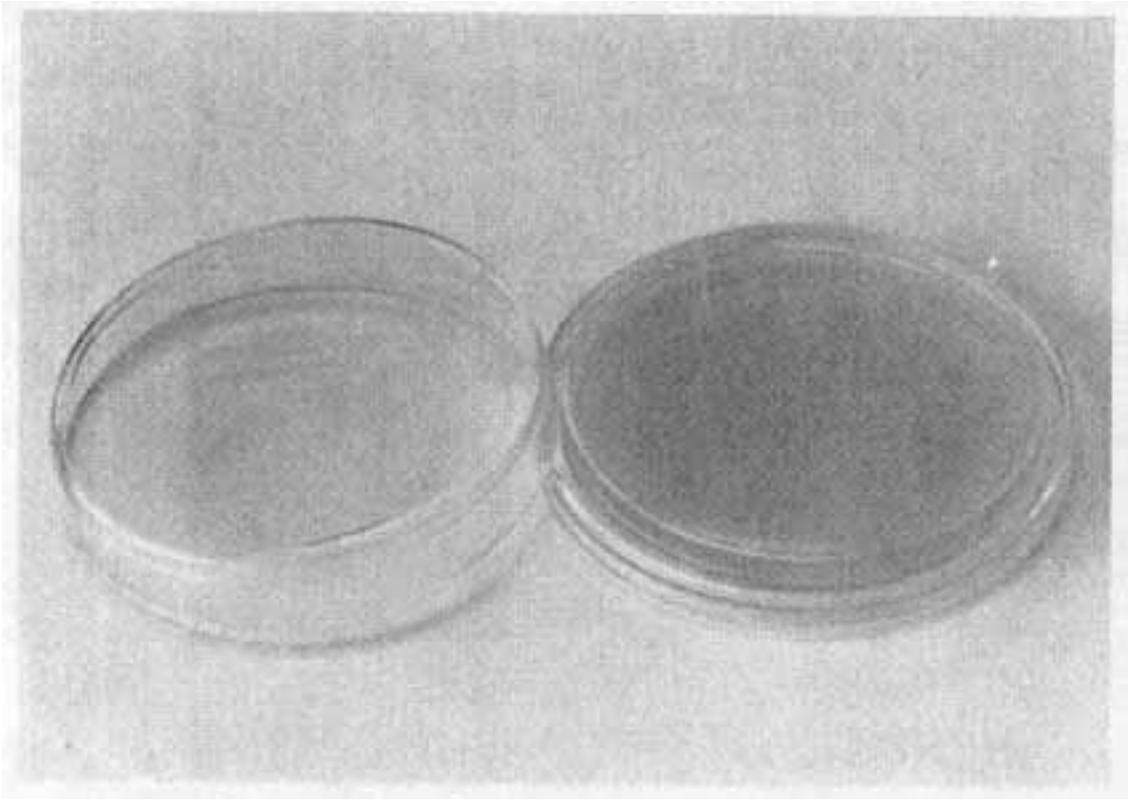

Fig. 1.15
Convex surface of Rodac agar plate.

Many of these environmental testing procedures and a suggested program for determining microbiological burden and action levels for both non-sterile and sterile environments can be found in a paper by Dell (83). Tables 1.17 and 1.18 are reproduced from his paper. 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.

The ultimate purpose of environmental control of microbial contamination is to minimize the potential for inadvertent product contamination. The lesser 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 passing a sterility test although

Table 1.17 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	Othera	Frequencyb
Walls and floors									
Sterility test area				X	X				M
Controlled areas				X	X				M
Critical areas				X	X				M
HEPA-filter 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

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

bM = monthly; B = batch or shift; H = history; D = daily.

Source: From Ref. 83.

Table 1.18 Example of Guidelines (Action Levels) for Environmental Monitoring of Sterile Production Elements

Sterile-processing locationa	Bacteria (per sq cm)		Mold (per sq cm)	
Controlled areas (Class 10,000100,000):				
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 areasair samplingb				
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 areasair samplingb				
Filling rooms	2	10	0	2
Sterility test laboratory	1	<1	0	0
Waterc		<1/ml	0/ml	

aControlled areas: sampled following sanitizing and prior to use.

bAir sampling: settling plate-exposed for 30 min or slit-to-agar sampling at a rate of 28.3 liters air/min for 30 min.

cWater tests same as for non-sterile manufacturing. Microbial count refers to at time use.

Source: From Ref. 83.

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 which 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 snap-shot 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 himself 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 laminar air flow workbench or other clean environment

4. Specific operations and manipulations while actually performing the sterility test, which are essential in maintaining asepsis

5. Proper clean-up 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 to be aware of when training people to work in sterile environments. These are listed in Table 1.19.

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 fragments 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 particulate-free gowning materials and adherence to strict gowning procedures will help to assure 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

Table 1.19 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 re-entering 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 intercomnever 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.

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.

Table 1.19 (Continued)

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 ran should be removed from the sterile room to avoid cross-contamination.

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.

contamination rate of admixtures compounded in a hospital pharmacy. What was important was that I.V. admixtures were compounded using a laminar air flow 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 which must be kept sterile. Procedure 8 in Appendix IV should be reiterated at this point before reading further. 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 he has accidently touched a sterile surface, 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 and, 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 responsibility 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 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 I.V. bottle (45), while the other method involves the filtration of all of the remaining portion of the bottle's contents 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, 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 contaminant, especially in large-volume solutions because of the high dilution factor. In the second method, a special device [Steritest, Millipore (Figure 1.16), or IVEX-Abbott (Figure 1.17)] is designed to permit in a closed system the

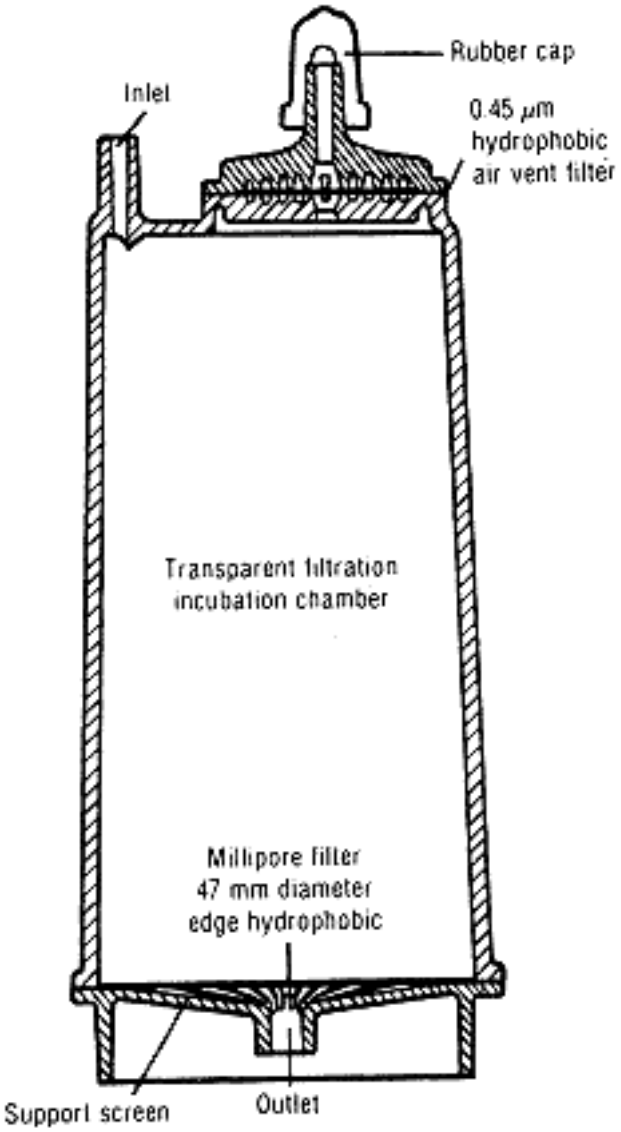

Fig. 1.16
Steritest chamber
(Millipore Corp., Bedford,
Massachusetts, from Ref. 78).

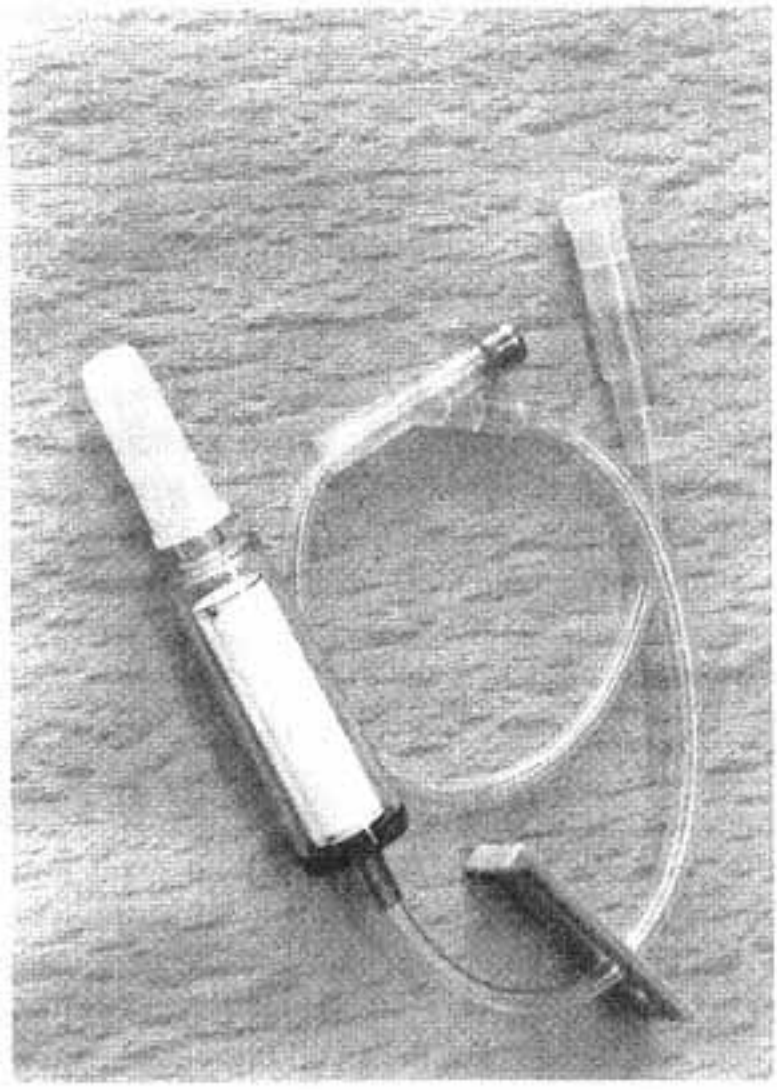

Fig. 1.17
IVEX-2 device (courtesy of Abbott Laboratories, North Chicago, Illinois).

filtration of the entire contents of a bottle through a plastic presterilized unit containing a 0.220.45 μm membrane filter. Trypticase soy broth is then added aseptically and the unit is incubated intact. This closed system was designed to reduce the rate of false positives and to 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 ml. Ten ml aliquots from each bag 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 I.V. 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 trypticase soy broth were found to be more reliable and sensitive than Addi-chek in detecting low-level bacterial contamination in I.V. 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 membrane filtration method for the sterility testing of antibiotics has been described (94). The modified unit is shown in Figure 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

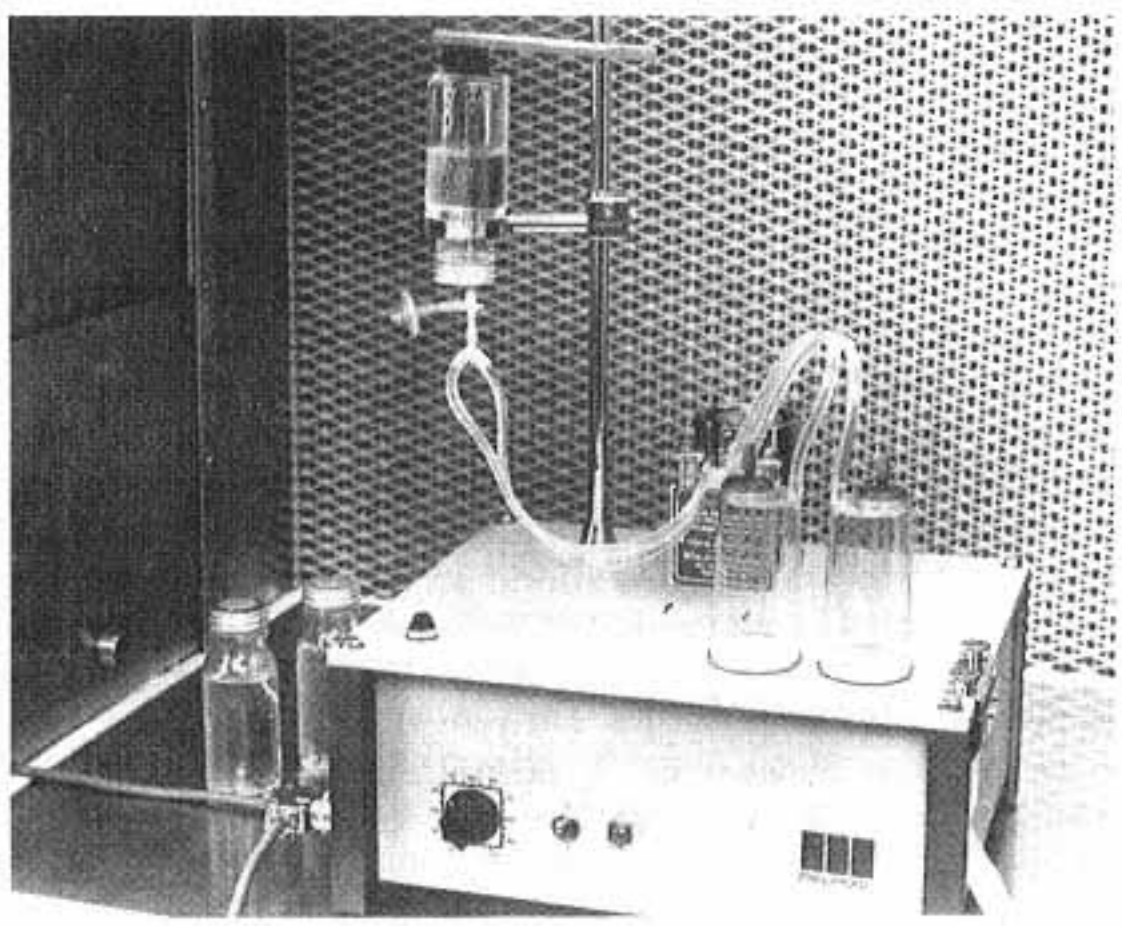

Fig. 1.18
Diagram of modified Steritest unit (from Ref. 84).

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 I.V. solutions and administration sets (under actual use conditions) was found to have valuable application in I.V. 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 I.V. 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) using a sterile filter unit like those already described. Ten percent of each lot of unit dose syringes 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 I.V. admixtures combines the membrane filtration 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 three minutes. The stain is removed by vacuum and the membrane 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 ml and the technique can be automated.

Several alternative methods of sterility testing have been suggested in 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 offered 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 14C substrates, and incubated for 25 days. The Bactec instrument automatically tests the vials by analyzing the atmosphere in the vials. If the vial contains microorganisms, they will metabolize the 14C substrates to product 14CO2. A positive result will be indicated once a threshold level of 14CO2 is exceeded. Models are available to test 60 culture vials per hour. The system offers expediency and convenience not characteristic of official sterility testing methods.

International Developments in Sterility Testing

Cooper (101) reviewed the international status of sterility test regulations in various European and Asian countries:

EEC Guide to Good Manufacturing Practice, 1989: de-emphasizes the role of the sterility test as it should be regarded as the last in the series of controls by which sterility is assured.

EUCOMED Recommendations for the Sterilization of Medical Devices, 1988: also de-emphasizes the role of the sterility test. This monograph promotes the use of parametric release for radiation and ethylene oxide sterilization and implies that the sterility test should be performed only if parametric release has not been validated.

International Federation of the Pharmaceutical Industries (FIP)Sterility Assurance Based on Validation of the Sterilization Process Using Steam Under Pressure, 1989: deals with the limitations of sterility testing of effectively terminally sterilized products. This document states that if adequate process control of steam sterilization is in place the product may be released with testing samples for sterility.

The International Pharmacopoeia, Third Edition, Tests for Sterility and Sterilization Methods: a World Health Organization monograph which differs from the USP Sterility Test in several areas including choice of microorganisms for testing the culture media, the quantity of microbial inoculum, and no details for performing bacteriostasis or fungistasis. WHO allows sterility retesting with no directions on investigation of initial sterility test failure.

The British Pharmacopoeia (BP) Test for Sterility (1988): very similar to the WHO test. Additionally the BP test allows two retests provided the organisms present in the first and second tests are distinguishable. No mention is made of investigating whether the retest was necessary due to inadvertent contamination in the testing environment.

The Pharmacopoeia of Japan (JP) Sterility Test (1986): very similar to the USP Sterility Test.

Code of Federal Regulations (CFR 21)1989FDA Sterility Tests: virtually lack any restrictions on performing retests (CFR will allow a second retest if initial and first retest could be showed to be invalid) and is less restrictive that the USP test which allows only one retest using twice the number of samples.

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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 solutions is especially serious because of the large amounts of fluid administered to people whose illnesses must be of the severity 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 bacteria(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

Table 2.1 Adverse Physiological Effects of Pyrogens in Humans

Primary

1. Increase in body temperature
2. Chilly sensation
3. Cutaneous vasoconstriction
4. Pupillary dilation
5. Piloerection
6. Decrease in respiration
7. Rise in arterial blood pressure
8. Nausea and malaise
9. Severe diarrhea
10. Pain in the back and legs
11. Headache

Secondary

1. Cutaneous vasodilation
2. Hyperglycemia
3. Sweating
4. Fall in arterial blood pressure
5. Involuntary urination and defecation
6. Decreased gastric secretion and motility
7. Penile erection
8. Leucocytopenia, leucocytosis
9. Hemorrhage and necrosis in tumors
10. Altered resistance to bacterial infections
11. Depletion of liver glycogen
12. Rise in blood ascorbic acid

13. Rise in blood nonprotein nitrogen and uric acid

14. Decrease in plasma amino acids

representation of the three cell wall layers of a gram negative micro-organism(1). The outer membrane shown in the figure is not found in gram positive bacteria. This structure contains the LPS moiety which interacts with the coagulable protein of the amebocytes of the horseshoe crab, a phenomenon from which evolved the *Limulus* Amebocyte Lysate (LAL) test.

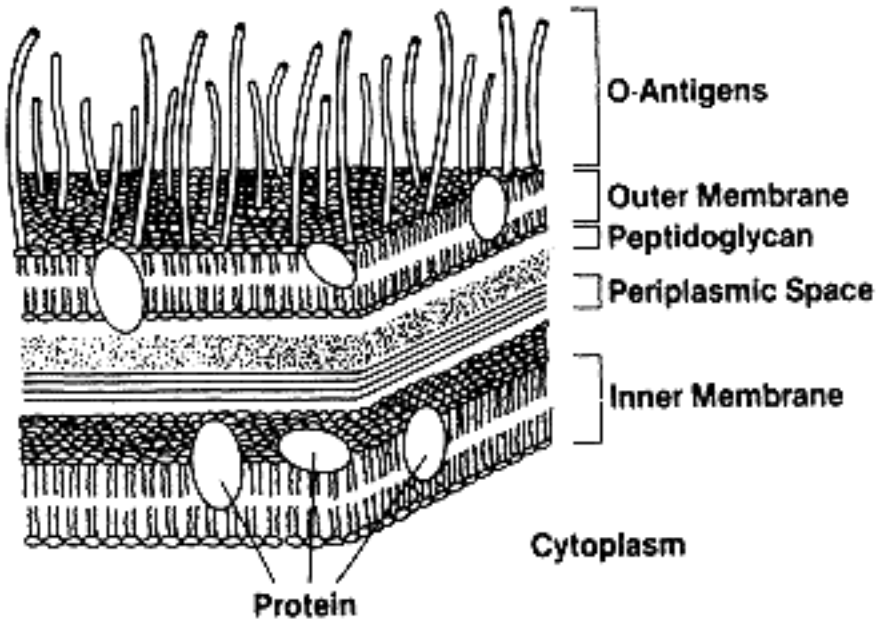

Fig. 2.1
Schematic representation of the three cell wall
layers of a gram negative bacterium (from Ref. 1).

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 β-hydroxymyristic acid replacing one of the amino hydrogens, and fatty acids replacing hydrogen in some of the -OH groups (see Figure 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, and processing equipment that have been depyrogenated initially, then employing correct and proper procedures

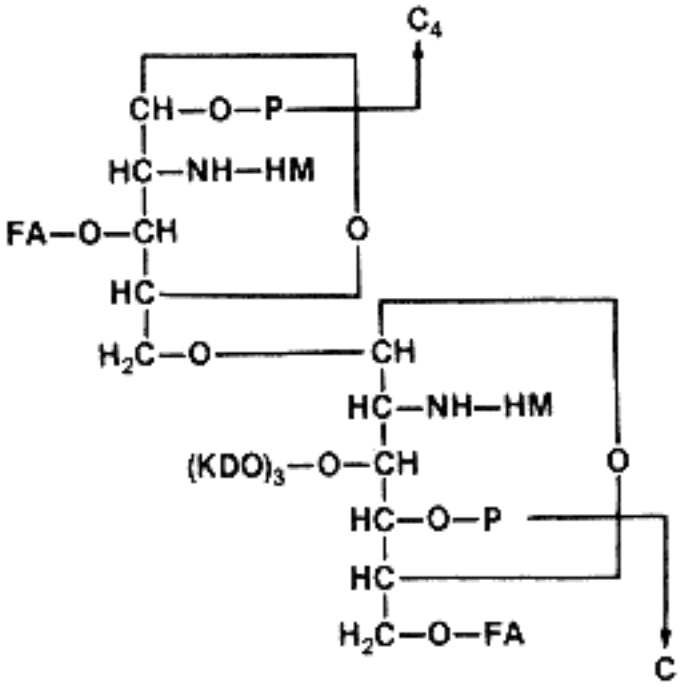

Fig. 2.2
Structure of unit of lipid A from *Salmonella* lipopolysaccharide. KDO: 3-deoxy-D-mannooctulosonic acid; HM: β-hydroxymyristic acid; FA: other long chain fatty acids (reproduced in part from Ref. 2 and Reitschel et al., *Eur. J. Biochem.*, 28, 166, 1972).

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 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 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. Additionally, bacterial pyrogens were not destroyed by autoclaving or removed by filtration.

It is interesting to note that while the medical significance of pyrogen was recognized during these years, it was not until 1923 that Florence Seibert(11,12) recommended that all pharmaceuticals be tested for pyrogens. Seibert's carefully controlled experiments confirmed Hort and Penfold's 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, 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 United States Pharmacopeia (USP) 12th edition. Later, in 1945, the Code of Federal Regulations* 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 unchanged. The LAL test for endotoxin, discussed later, became an official USP test in 1985. Today the LAL test, or more commonly called the Bacterial Endotoxin Test, has pre-empted the rabbit test as the USP method of choice for detection of endotoxin in

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

parenteral products. Today, 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 or pharmaceuticals and medical devices, and end-product evaluation of devices, 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, the content of this section will follow closely both the specifications written in the 22nd edition of the USP(16) and the excellent review article written in 1973 by Personeus(17).

General Description of the USP Pyrogen Test

The following paragraph is quoted directly from the USP 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 intravenously 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 apparatusesglassware, containers, syringes, needles, etc.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 30 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

The USP 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 period 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 which 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 millivolts. Common thermocouple types are listed in Table 2.2. Typical thermocouples are composed of three parts as shown in Figure 2.3. The two dissimilar wires are supported by an electrical insulator, either hard-fired ceramic or non-ceramic materials such as Teflon, polyvinyl chloride, fiber glass, fibrous silica, or asbestos. The outer sheath can be composed from a variety of materials, most commonly stainless steel, Teflon, and various elemental metals (platinum, copper, and aluminum).

Thermocouples must be accurately calibrated against National Bureau of Standards (NBS) 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 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 ±0.1°C of the calibration bath temperature or should not be used in pyrogen testing.

Rabbit body temperature data are recorded electronically by instruments such as those seen in Figure 2.4. Electronic temperature recorders usually can monitor over 100 rabbits simultaneously. Any variation in

Table 2.2 Commonly Used Thermocouple Types

Type B	Platinum-30 percent rhodium (+) versus platinum-6 percent rhodium (-)
Type E	Nickel-10 percent chromium (+) versus constantan (-)
Type J	Iron (+) versus constantan (-)
Type K	Nichel-10 percent chromium (+) versus Nickel-5 percent (-)
Type R	Platinum-13 percent rhodium (+) versus platinum (-)
Type S	Platinum-10 percent rhodium (+) versus platinum (-)
Type T	Copper (+) versus constantan (-)

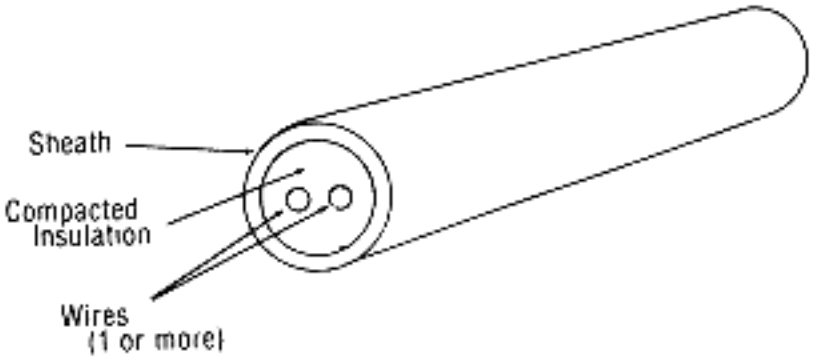

Fig. 2.3
Composition of a typical thermocouple used
in rabbit pyrogen testing (courtesy of the American Society
for Testing and Materials, Philadelphia, Pennsylvania).

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

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 are now available for automatic temperature recording during pyrogen testing. 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. 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 one to two 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 both in the rectal insertion of the thermocouple and the injection of the test product.

The normal basal body temperature of rabbits ranges between 38.9 and 39.8°C (102.0103.6°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 upon 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 re-establishment of the true normal body temperature.

Rabbits may become tolerant to pyrogenic activity after repeated injections of endotoxin(2022). 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 two weeks.

Test Procedures

The USP procedure recommended for performing the pyrogen test is reprinted 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 example, 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, 70 ± 5°F. Housing should be individual cages designed to maintain cleanliness (see Figure 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 actual pyrogen testing. Rabbits in restraining boxes (see Figure 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 which is closed during the

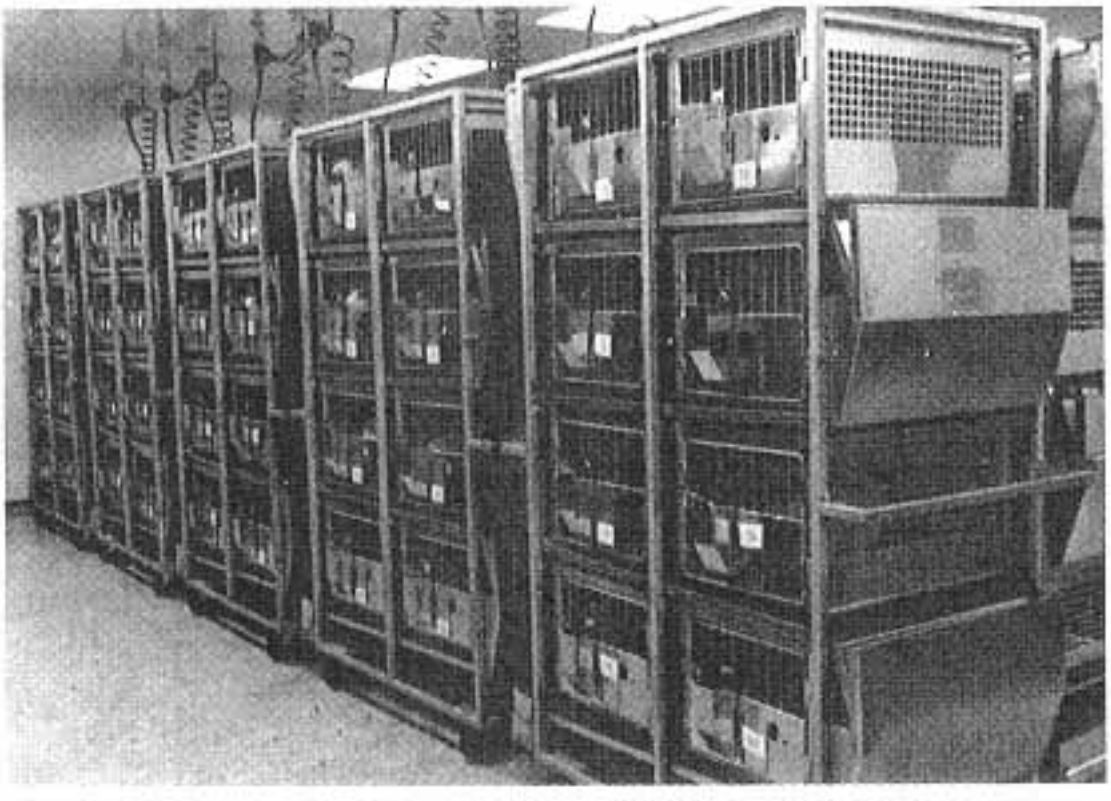

Fig. 2.5
Housing of pyrogen test rabbits in clean, individual cages.

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 0.21.0°C rise in body temperature which may not return to normal for 6090 minutes.

During the pyrogen test, which could last four to six 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. Examples of modern restraining boxes are shown in Figures 2.6 and 2.7.

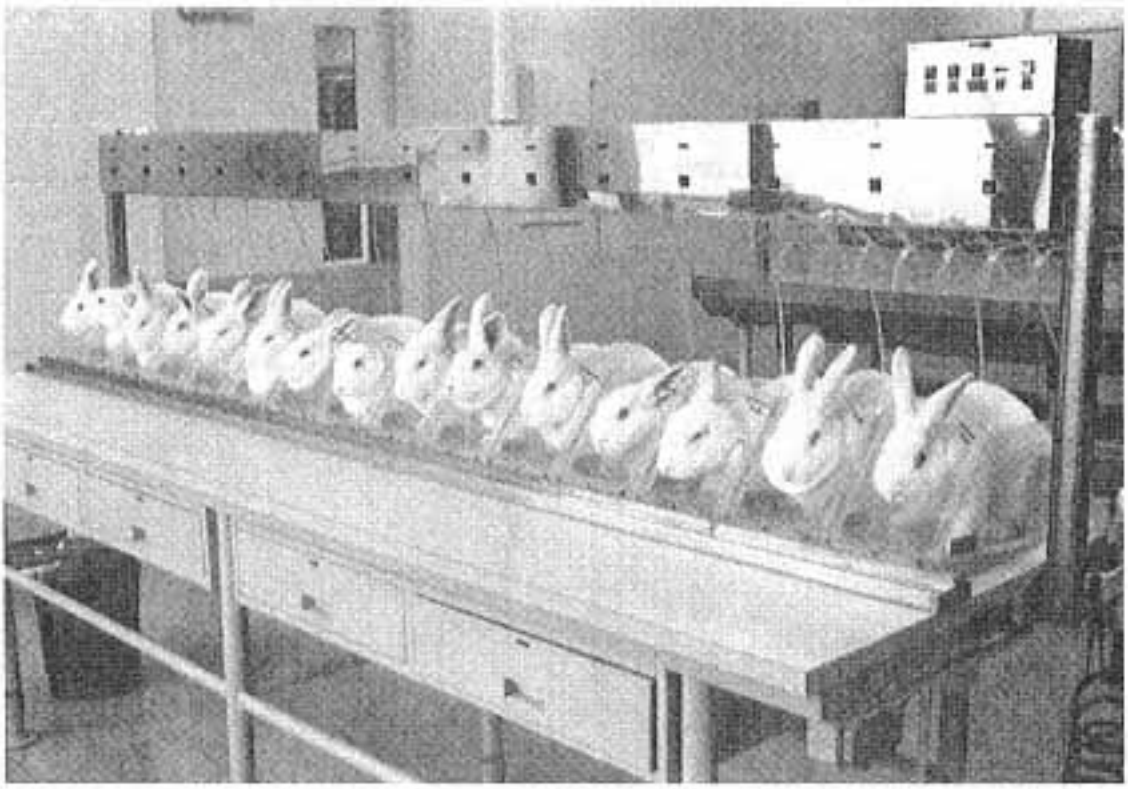

Fig. 2.6
Rabbits situated in individual restraining boxes.

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 Figure 2.7) are inserted in the rectum to a depth of not less than 7.5 cm. Following a 3045 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 2023 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, Phytonadione Injection, USP, 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

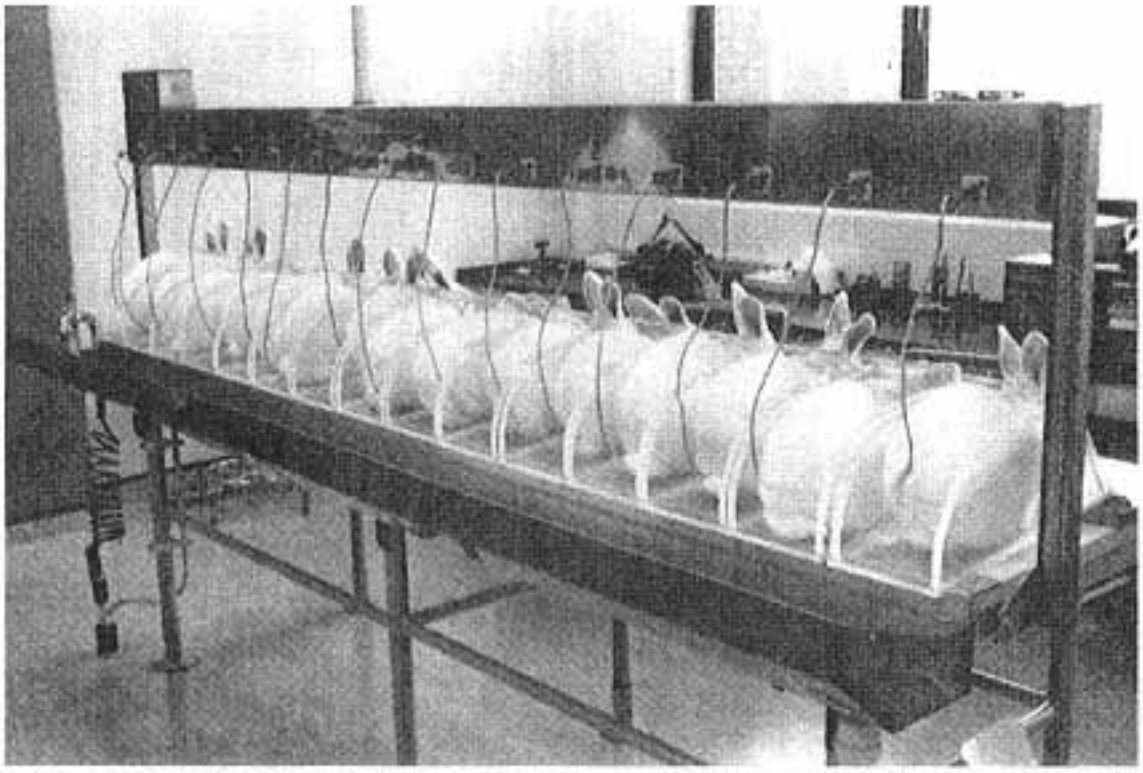

Fig. 2.7
Rear view of rabbits in restraining boxes.

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 the following:

1. Rest the ear against the fingers of the left hand and hold the ear down with the thumb (see Figure 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 back pressure will be felt. Withdrawing the needle slightly and moving it forward again should place it in proper position.

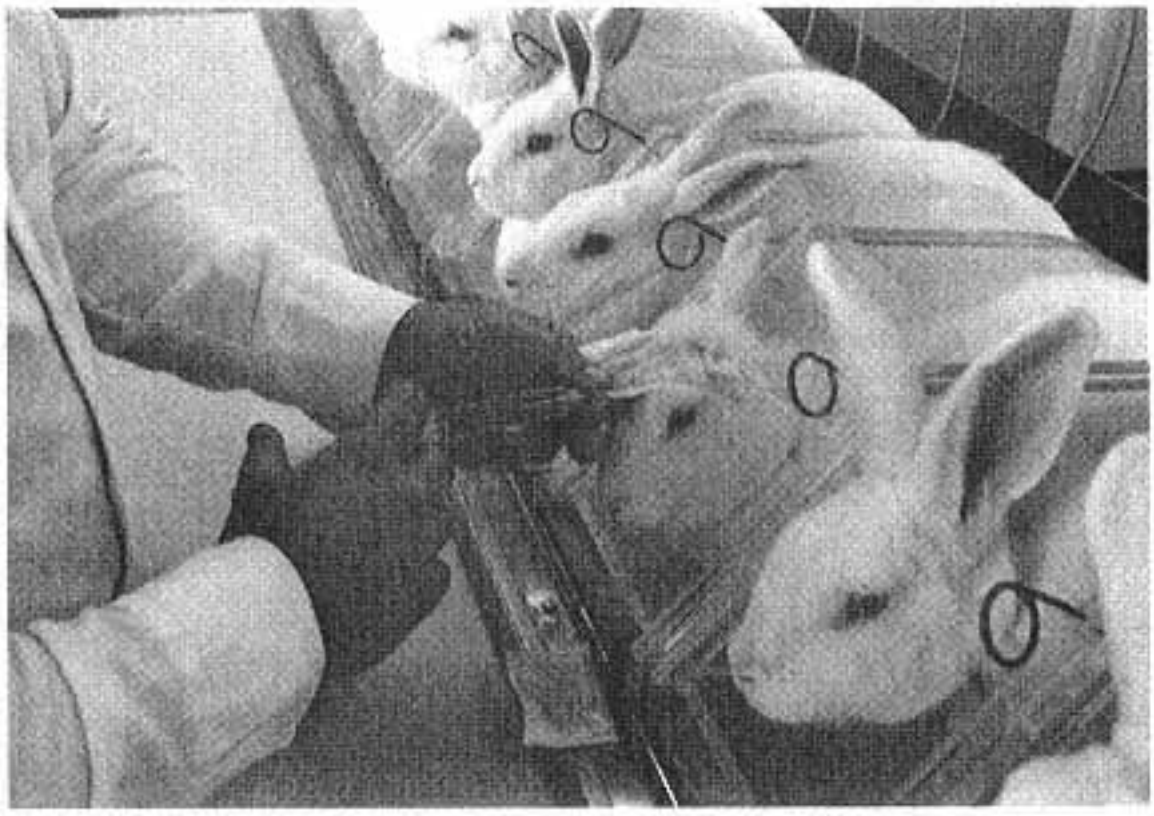

Fig. 2.8
Injection of pyrogen test sample into ear vein of rabbit.

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 one, two, and three 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 InterpretationUSP

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 showed individual temperature rises of 0.5°C or more.

The United States Public Health Requirements for Biological Products, Part 73, judge a solution to be pyrogenic if at least half 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(24) pyrogen test employs a sliding scale based on three rabbits and additional groups of three 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 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 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

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

	Maximum total peak response (°C) to pass the test		Minimum total peak response (°C) to fail the test	
Number of rabbits	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

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 kg 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 (pyrogenic) the test at ≤1.0 ng per ml doses (10 ml/kg of 10 ng/kg endotoxin) of *E. coli* 055:B5

Table 2.4 Eight Rabbit Pyrogen Test Results in Saline with *E. coli* 055:BS Using 35 kg Rabbits

E. coli endotoxin concentration (ng/ml)	Volume solution injected (ml/kg)	USP total temperature increase (°C)	Mean temperature increase (°C)	Standard deviation (°C)	Coefficient of variation (%)
3.125	1.0	7.80c	0.975	0.246	25.2
1.56	1.0	4.75c	0.594	0.218	36.7
1.00	1.0	3.70c	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

aNegative rabbit temperature values were excluded from total temperature increase determinations according to USP.

bNegative rabbit temperature values were included in the determinations of means and standard deviations to properly reflect total variability.

cFailed USP test criteria of 3.7° total increase.

Source: Ref. 25.

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 out of 12 laboratories conducting rabbit pyrogen tests, four passed a level of 2.5 ng endotoxin per kg level(26).

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

Table 2.5 Examples of Drugs and Drug Products$crNot Suitable for Testing by the USP Pyrogen Test

1. Most cancer chemotherapeutic agents
2. Most anesthetics, muscle relaxants, and sedatives
3. Sterile Betamethasone Sodium Phosphate Solution
4. Chlorpheniramine injection
5. Magnesium sulfate
6. Metocurine iodide injection
7. Perphenazine
8. Thiopental sodium for injection

products are inherently toxic to the rabbit (see Table 2.5) and must be diluted to concentrations far below the pharmacologically effective dose of the drug.

Despite these major limitations and the insurgence today of the *Limulus* Amebocyte Lysate (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.

The LAL 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 upon 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 horseshoe crab's 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, to be discussed in the following section, LAL 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 Figure 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 cells. Carefully performed, this procedure is not fatal to the crab, and upon proper restoration, the crab can be used again. Since amebocytes act as activators of the coagulation mechanism in the crab, an anti-aggregating 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). Upon reconstitution LAL has a shelf life of one month's 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, Inc., 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 FDA stated that LAL was a biological product, thus 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 later that year (September 18, 1973; 38 FR 26130). In the ensuing years, available data on and experience with the LAL test

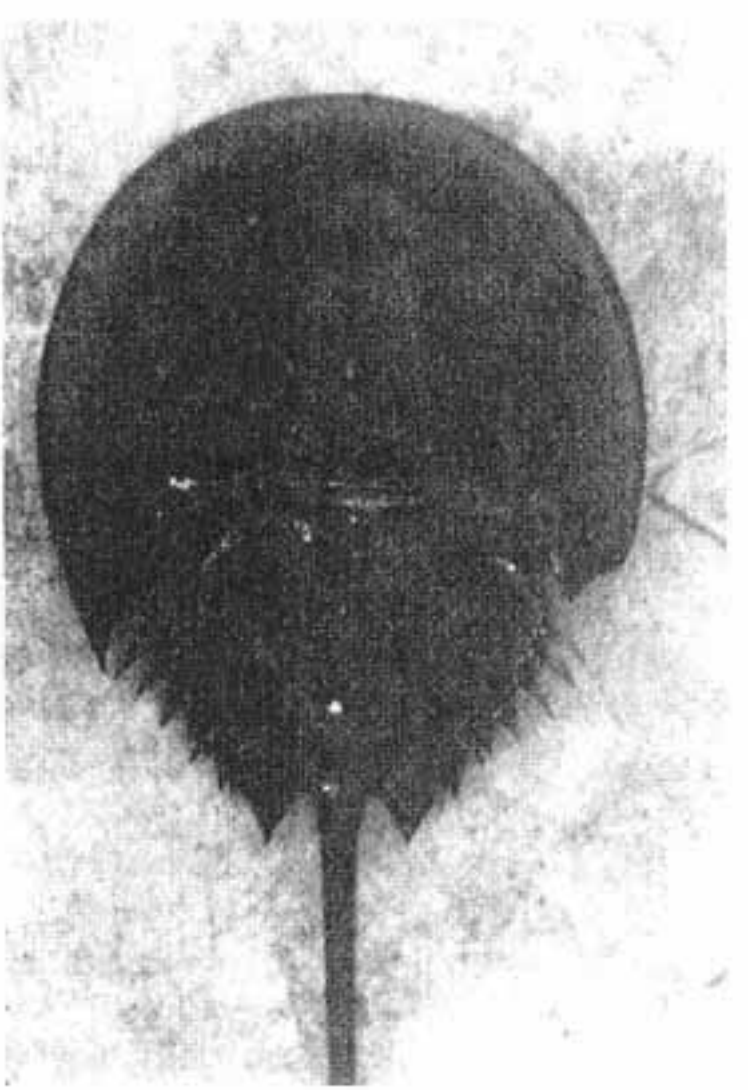

Fig. 2.9
Limulus polyphemus, the source of
Limulus amebocyte lysate reagent.

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 devices 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).

LAL 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).

*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

1. Use of United States Standard Endotoxin for determining the sensitivity of LAL.

2. Use of United States Reference LAL for establishing the potency of LAL.

3. 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.

4. 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.

5. Various labeling requirements.

6. 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.

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 = 19,00025,000) contained also in the LAL substance.

4. The lower molecular weight fraction, called coagulogen, is cleaved by the proenzyme into a soluble and insoluble sub-unit. The insoluble sub-unit appears as a solid clot, a precipitate, or a turbid solution, depending on the amount of insoluble coagulogen by-product formed.

Therefore, the coagulation reaction requires three factors in addition to endotoxin. These three factorsa clotting enzyme, clottable

protein (coagulogen), and certain divalent cationsare found in the LAL reagent. A schematic representation of the LAL reaction mechanism is found in Figure 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 simply procedure, especially when 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 of 3638°C.

3. The pH of the reaction mixture must be within the range of pH 57.

4. The reaction time should be no longer than one 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 one 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

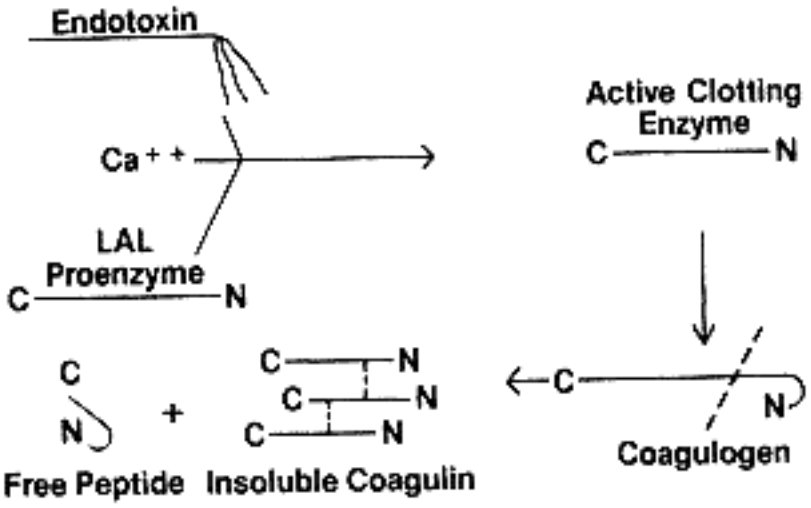

Fig. 2.10
Schematic representation of the LAL reaction mechanism (from Ref. 41).

its integrity after slow inversion of the test tube containing the mixture (see Figure 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 bacterial endotoxins in sample materials. These instructions will be summarized with commentary below:

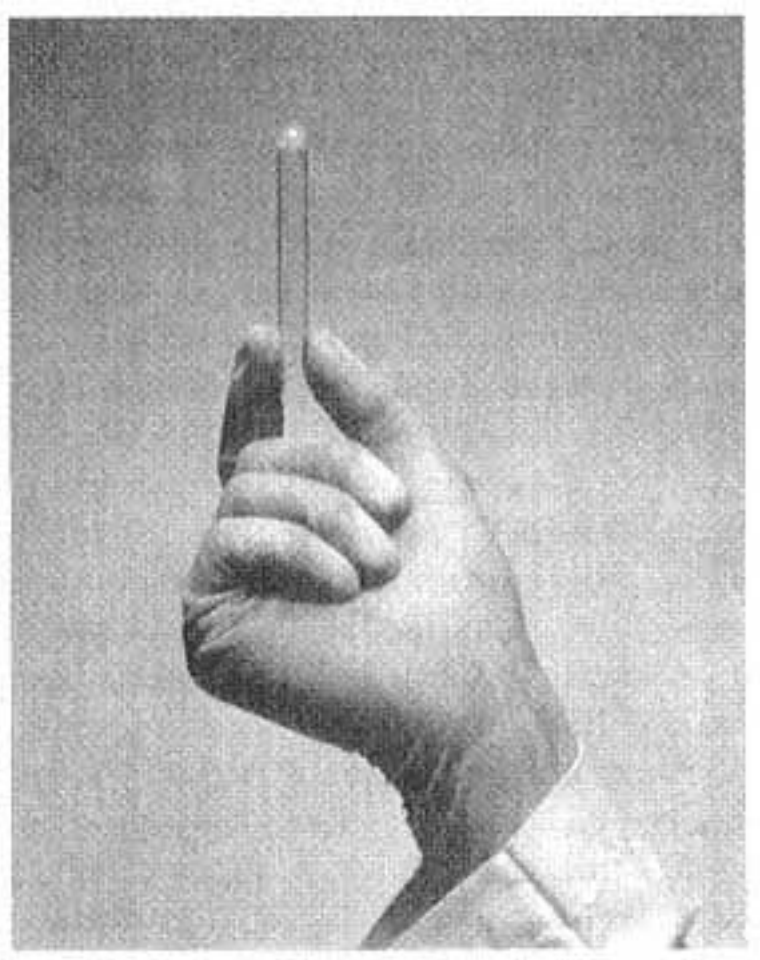

Fig. 2.11
A positive LAL gel-clot test is characterized
by the formation of a solid gel which remains intact
in the bottom of the tube upon inversion
(courtesy of BioWhittaker,
Inc., Walkersville, Maryland).

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 away 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 four 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 one Endotoxin Unit (EU)* in 0.2 nanograms (ng) of the standard(43). The current FDA and USP reference standard is purified lipopolysaccharide from E. coli 0113. One vial contains 10,000 EUs.

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 endotoxin standard (CSE) against the USP RSE.

There are seven 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

*It has become accepted practice to use Endotoxin Units (EU) as the more desirable expression of endotoxin strength than weight or concentration terms. The use of EU 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 will use the EU term as much as possible, but most literature references cited will use the weight or concentration terms as reported in the published articles.

USP Endotoxin Standard (Lot F). The FDA tests each lot for potency before releasing it to be marketed.

If a manufacturer chooses to use an endotoxin preparation (CSE) other than the U.S. Reference Standard Endotoxin (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 4 vials of the lot of CSE should be assayed by determining endpoints (gelations) with LAL. The values obtained should be the geometric mean of the endpoints using a minimum of four replicates.

2. The endpoint for the CSE is stated in ng per ml. The endpoint for the RSE is EU per ml. So, if the LAL endpoint for the CSE is 0.018 ng/ml and the LAL endpoint for the RSE is 0.3 EU/ml, then:

RSE = 0.3 EU/ml = 16.6 EU/ng of CSE

CSE 0.018 ng/ml

3. This indicated 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 endpoint, chromogenic and endpoint-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 (sensitivity). 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 are prepared by twofold dilutions of the RSE or CSE endotoxin using LAL-negative water for injection. Following incubation and endpoint 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 are 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 endpoints (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

$$\frac{\Sigma\ \text{E (endpoints)}}{\text{f (number of replicates)}}$$

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 control 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.

*The FDA validation guideline contains specific directions for inhibition/enhancement testing depending on the technique usedgel clot, inorganic and endpoint turbidimetric, and kinetic turbidimetric.

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

MVC Determination

$$MVC = \frac{\lambda M}{K}$$

λ = Sensitivity of LAL reagent in EU/ml

M = Rabbit dose or maximum human dose/kg

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:

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

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

MVD Determination

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

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

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

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.

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

Replicates (f)	Gel endpoint results for specimen dilutions: Unity	0.5	0.25	0.125	Endpoint dilution factors (E)
1	+	+	+	-	0.25
2	+	+	-	-	0.5
3	+	+	-	-	0.5
4	+	+	+	-	0.005:0.25
5	+	+	-	-	0.5
					$\Sigma E = 2.0$

[a]Geometric mean $= \frac{\Sigma E}{f} = \frac{2.0}{5} = 0.4$

Source: Ref. 41.

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 long as the 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 than 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. Borosilicate 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 2040% 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 or 0.1 N hydrochloric acid or suitable buffers.

Test tubes, usually of the dimensions 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 + 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 ± 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 degrees) 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 Figure 2.11.

Instrumental Tests

Direct visual observation of the gel endpoint 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 endpoint by instrumental methods offers several advantages, including single-tube quantitation and objectivity. Additionally, 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 Bio Whittaker).

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

4. The OD values are recorded on a cassette tape and/or paper and can be displayed graphically as OD vs. time on a cathode ray tube or transferred to paper with a hard copy printer. An example of a plot of

OD at 670 nm vs. time using standard endotoxin samples is shown in Figure 2.1(49).

Turbidimetric optical density has been shown to be directly proportional to *E. coli* endotoxin concentration on a log-log plot. For example, Figure 2.13 shows such a relationship. Standard curves usually are linear only within a relatively small concentration range, for example, 0.010.1 ng/ml (0.11.0 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.

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

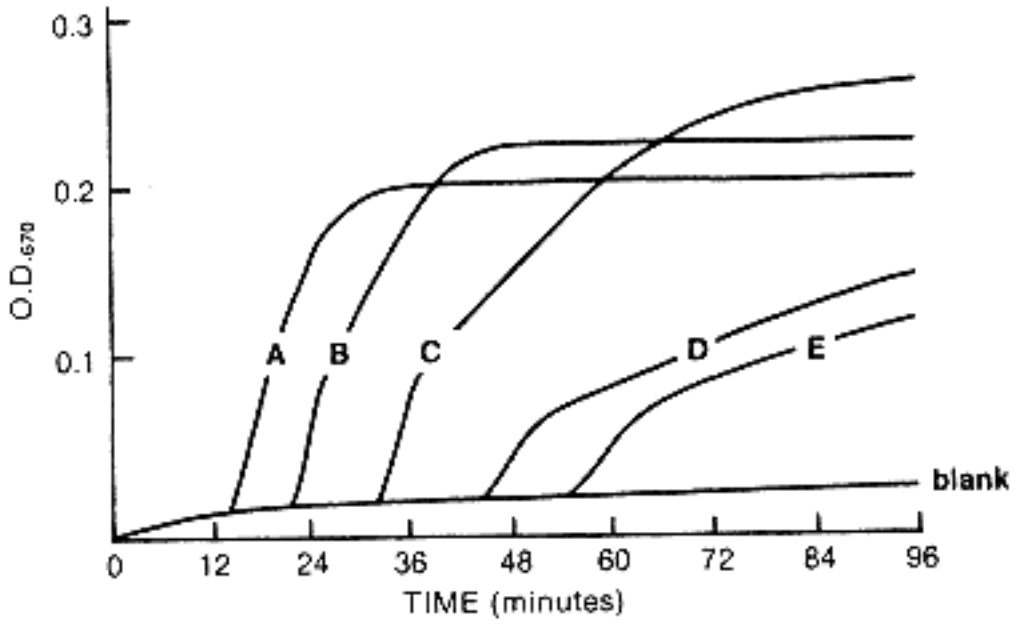

Fig. 2.12
Turbidimetric response of LAL with control standard endotoxin diluted in sterile water for irrigation. A, 100 pg endotoxin/nl; B, 25 pg/nl; C, 6.3 pg/nl; D, 1.6 pg/nl; E, 0.4 pg/nl (from Ref. 49).

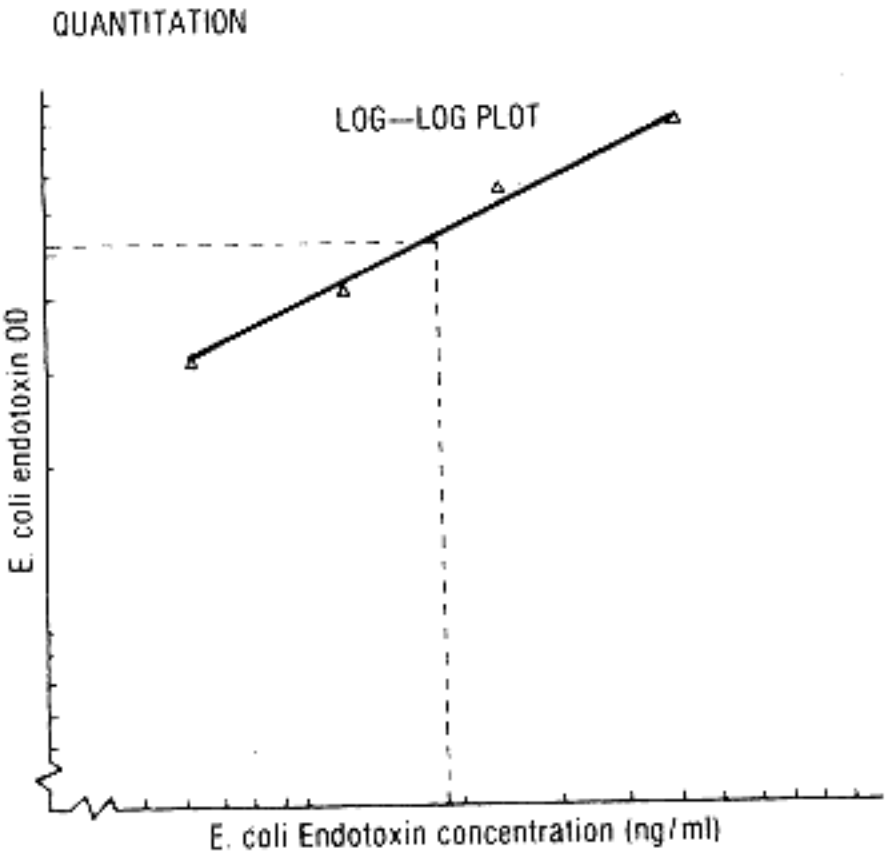

Fig. 2.13
Log-log relationship between turbidimetric optical density and endotoxin concentration (courtesy of the Millipore Corporation, Bedford, Massachusetts).

blood which 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 endpoint. This system is called the Chromogenic LAL assay system (Figure 2.14). 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 three 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. Endotoxin concentration can then be

Fig. 2.14
The latest development in LAL technology is the
kinetic chromogenic method, one instrument of which is shown here
(courtesy of Bio-Whittaker, Inc., Walkersville, Maryland).

determined from a standard plot of absorbance vs. endotoxin concentration in ng/ml or EU/ml.

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 124125. The activated enzyme, in turn, catalyzes the splitting of pNA from the colorless substrate. In Figure 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 where 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 endotoxin units per 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 as well as being 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.

Appendix V at the end of this book presents a comparison of published LAL methodology courtesy of a review article by Novitsky(56).

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 where 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. Additionally, 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 expected to be present in all articles at some level. The question iswhat 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) that FDA accepted and became part of the new FDA draft guideline for end product testing published in March, 1983(60). The new endotoxin limit is

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

where the TPD has been defined as 5 EU/kg, the lower 95% confidence limit of the average does found to produce a pyrogenic response in rabbits and humans(61). For drugs administered intrathecally, where pyrogenic contamination can be much more dangerous (see pages 154155), the TPD is 0.2 EU/kg.

The maximum rabbit or human dose is that dose administered per kg of body weight of rabbit or man in a single hour period, whichever is larger. For example, if a drug of 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 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 license where 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 kg and the corresponding endotoxin limits for a large number of aqueous injectable drugs and biologics on the market. The 5th 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, which will remain intact when inverted carefully after one 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 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 was the rabbit test. This was later confirmed by Elin and Wolff(63).

Improvements in LAL production and formulation methodology increased the sensitivity of LAL 10 to 50 times greater than the rabbit test(42,64). These numbers were based on a gel time of one 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 the lower sensitivity of the rabbit test had to be compensated for by injection of a higher volume of test sample.

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 ml 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 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.

Table 2.9 LAL Specificity and Sensitivity for the Detection of Lipopolysaccharides

LPS source	Minimum dose for positive rabbit responsea	Minimum conc. for positive LAL reactionb
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

aThree rabbits used.

bLAL source: Pyrogent (Mallinckrodt).

Source: From Ref. 65.

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 testa	Limulus test
500	Pyrogenic	Positive
50	Pyrogenic	Positive
5	Nonpyrogenic	Positive
0.5	Nonpyrogenic	Positive
0.05		Negative
0.005		Negative

aDose: 1 ml endotoxin solution/kg body weight.

Source: From Ref. 66.

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 endpoints varied among the LAL reagents from the different manufacturers. Lot-to-lot variability with LAL reagents from two manufacturers was observed as well as 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 mcg/ml endotoxin in plasma whereas 0.05 mcg/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 will be discussed in the section Limitations of the LAL Test.

LAL 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.

Amebocyte lysate contains substances called (13) beta-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 beta-glucan (11000 ng/ml plasma) will trigger gelation while greater amounts of beta-glucan (1 mg/ml plasma) will not(76).

Person 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 positives 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 of the LAL Test 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 expensive

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 are 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, drugs designed for intrathecal injection, and validation of dry heat depyrogenation cycles. These applications will be 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:(1) employing an in vitro test, when available, rather than an animal test;(2) results are available within 90 minutes after beginning the test procedure;(3) tests 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 Jirillo(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 large-volume parenterals (LVPs)(25,3032). 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 not 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 this author 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 itself as an excellent alternative or supplemental method to 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). Seven of the 10 quality control representatives from the parenteral industry polled by the author identified inhibition of the lysate-endotoxin interaction as the number one factor limiting the applicability of the LAL test. As discussed on pages 124125, the LAL gelation reaction is mediated by a clotting enzyme that is heat labile, pH sensitive, and chemically related to trypsin(8990). 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

Table 2.11Examples 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. Parenteral Sci. Tech., 38,* 190201, (1984).

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 endpoint of detection for the product sample should be no different from the end-point for the standard 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 of LAL Test.

According to Cooper(90) 30% of drug products do not inhibit the LAL test (producing an increase in the expected gelation onset time). O the majority of products which do inhibit the test 97% of the problems can be resolved because the inhibition is concentration dependent. Simple dilution usually can overcome inhibitory properties of drug products against the LAL-endotoxin reaction.

LAL test inhibition is considered significant if the positive control varies by more than a two-fold 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).

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

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:

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 upon 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 beta-glucans.

8. Extensive studies are required to validate the LAL test as the final product pyrogen test.

LAL 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 Reference Standard Endotoxin, 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 already discussed on page 133 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 Guideline on Validation of LAL Test as an End Product Endotoxin Test of Human and Animal Parenteral Drugs, Biological Products, and Medical Devices(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 *Escherichia coli*-delivered LPS such as the USP Reference Standard Endotoxin 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 endpoint.

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. Biologicals

3. Devices

4. Disease states

5. Food

6. Validation of depyrogenation cycles

The literature is massive with regard to LAL test applications in most of these areas. Not all published reports will be discussed, but those with significant impact will be described in the following subsections. 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 product release test for pyrogens. Approximately 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 (99mTc), which has a biological half-life of only six to seven 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 radio-pharmaceuticals. 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 economic, 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 radioactive 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 medications for which the LAL test provides marked advantages 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 contains 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 $\leq$ 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 antitumor 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 non-inhibitory conditions for successful application of the LAL test. Case et al.(101) tested 28 antibiotics with the LAL assay to determine their non-inhibitory 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 a NIC 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 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 large-volume parenterals(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(1) dyes such as methylene blue and fluorescein for detecting cerebrospinal fluid (CSF) leakage,(2) contrast media for visualization of CSF pathways,(3) cancer chemotherapeutic agents such as methotrexate for treatment of leukemic meningitis,(4) antibiotics such as gentamicin for septic meningitis, and

(5) radiopharmaceuticals for radionuclide cisternography, a procedure wherein 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 co-workers (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.

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 his 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 alpha-globulins(110) and a serum globulin esterase(111).

Table 2.12 Comparison of LAL and Rabbit Tests on Various Materials

Material	No. of samples	Dose rabbit ml/kg I.V.	Rabbita		LALb 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	0.510	24	21	26	21	2
Total	345	0.510	127	258	123	224	45
Plasma human treated chloroform	28	1	18	10	5	23	

aRabbit + LAL - = Plasma 13.

bLAL : Pyrogent.

Other samples: 4.

Source: Ref. 65.

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 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 lipopolysaccharide 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 LAL 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 lipopolysaccharide 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, NCDRH) 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 non-pyrogenic 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 manufacturer 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 Figure 2.15. The collaborative study conducted under HIMA auspices evaluated the pyrogenicity of *E. coli* 055:B5 from Difco Laboratories (Detroit). Using 12 rabbit 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 eight rabbits were administered this concentration of endotoxin, four 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).

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

Two devices for lots of less than 30

Three devices for lots of 30100

3% of lots above 100 up to a maximum of 10 devices per lot

The recommended volume of non-pyrogenic rinsing fluid per device is 40 ml. If 10 devices are rinsed, then the total rinsing extract pooled is

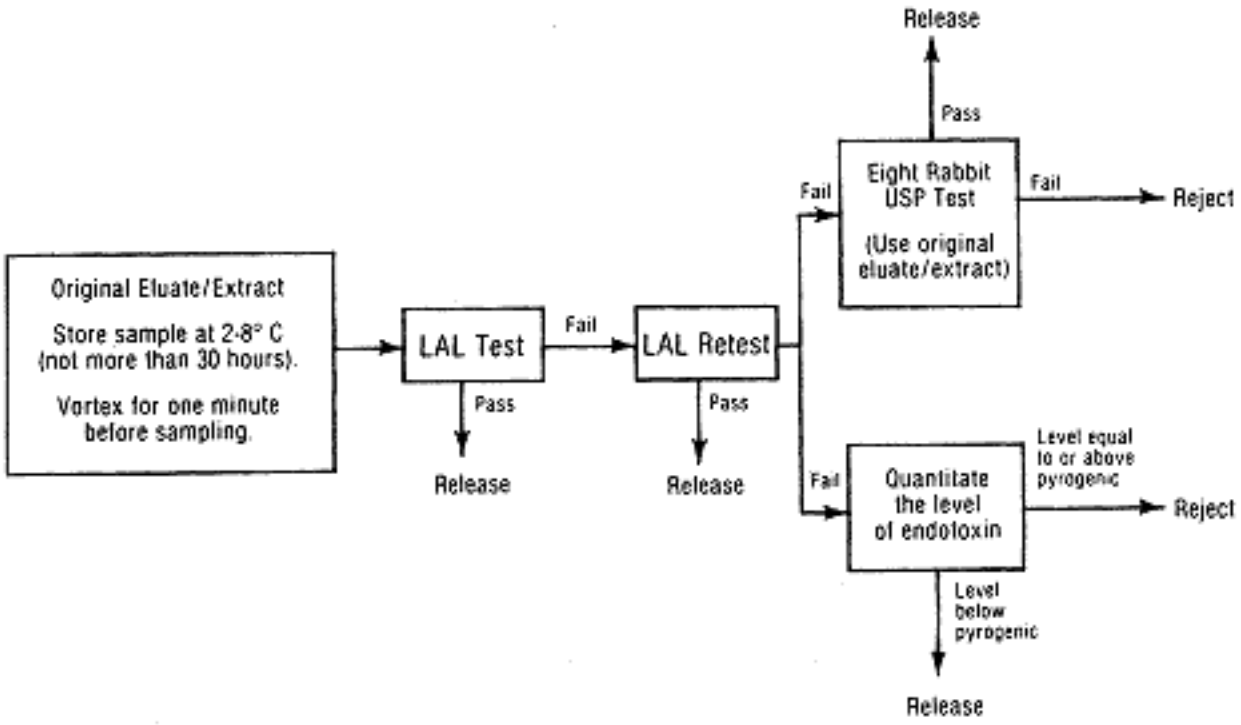

Fig. 2.15
Schematic flow chart of the guidelines proposed by HIMA for LAL pyrogen testing of medical devices (from M. Weary and F. Pearson, Pyrogen testing with *Limulus* amebocyte lysate, *Med. Device Diag.*, Nov. 1980, Cannon Communications, Inc.).

400 ml. If a rinse volume greater than 40 ml per device is required, a more sensitive LAL endpoint 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

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

simple, rapid, and reliable method for detecting endotoxemia. Early detection permits early and vigorous treatment of the infection in which the LAL test can be used to monitor the effectiveness of the treatment.

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. 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 (123127). 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 on 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(131132), postanasthia hepatitis(133), urinary tract infections(134), mastitic milk(135), gram negative sepsis resulting from burns(136) and other causes(137), gonococcal cervicitis in women(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 $\leq$ ng/g endotoxin for both white and beet raw sugar while 100 ng/g endotoxin was 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 a FDA

requirement for a 3-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 Pyrogen

Ultrafiltration, using a membrane having a fraction molecular weight of 10,000, has been used to separate endotoxin contamination 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, can cause more frustration 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 United States 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 particulate matter content and analysis in large-volume injections for single-dose infusion and in small-volume parenterals (2).

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 current particle testing methods in the quality control analysis of parenteral solutions. Two primary methods of particulate analysisvisual inspection and electronic particle countingwill be discussed in detail. Groves' textbook (3) has been used as a major resource of information contained in several of the sections of this chapter.

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 (4) 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 (5) 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 particles having sizes of approximately 50 μm came from a report by Brewer and Dunning (6). 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 (7).

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 (8) and cellulose (9). Glass particles and their potential hazard was studied by Brewer and Dunning (6) and later by Gradinger (10), but no evidence of foreign body reactions in animals were found. These and other reports led to the classic work done by Garvan and Gunner published in 1963 and 1964 (11,12). 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 post-mortem 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 on the analysis and potential clinical hazards of particulate matter that continue to this day.

A collaborative study conducted by the Pharmaceutical Manufacturers Association (13) involved the intravenous injection of varying quantities and sizes of inert polystyrene spheres into hundreds of rats, then performing necropsies at various periods of time from one hour to 28 days following injection. The results were as follows:

1. Thirteen of 18 rats injected with 8 × 106 particles per kg at a particle size of 40 μm died within five minutes.

2. Rats showed normal blood studies, organ weights, and pathologic criteria after being injected with either 8 × 106 particle size 0.4 to 10 μm or 4 × 105 particles per kg of particle size 40 μm.

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 ampuls (14). Large particles (> 20μm) were retained mostly in the lungs of mice while smaller particles (510 μm) were found in the liver, spleen, and kidney. No glass particle was 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 (15). 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. (16). 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 (17) have supported this finding.

Particles greater than 7 μm in diameter are viewed to be more threatening than particles of smaller size (18). Pulmonary capillaries are approximately 7 μm in diameter. Thus, theoretically, a particle greater

than 7 μm can be trapped in the vascular bed, resulting in multiple pulmonary infarctions.

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 manufacturerlack of particulate matter indicates good production technique and a high quality product.

2. Significance to the userlack 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 significancelack 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 (19).

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

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. I.V. administration sets

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

(20) and McCrone (21). It is imperative that particles seen in solutions not have originated during the particle measurement and identification procedures.

Particulate Matter Standards

The first reference to particulate matter in the USP occurred in the eighth edition in 1905 (22,23). Diphtheria Antitoxin, a hypodermic injection product, was described as a transparent or slightly turbid

Table 3.2 Type and Approximate Size Range of Some 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. 3.

liquid. Not until 1936, in the National Formulary (NF), sixth edition, was the term clearness defined for parenteral products (24): 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 or fibers, or cotton hairs, or any undissolved material.

The words substantially free caused interpretative 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 of 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 twenty-second 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. Visible has the connotation of particles being seen with the unaided eye. The unaided eye can discern, at best, particles at sizes of about 4050 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 middle 1970s the USP and FDA co-sponsored 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 LVPs. Ultimately, the efforts of this subcommittee resulted in the establishment of the USP microscopic assay procedure

for the determination of particulate matter in LVPs for single-dose infusion and set upper limit acceptable particle standards at particle standards at particle sizes of 10 μm and 25 μm (25). These two sizes were also subsequently used as size standards for particulate matter in small-volume injections.

The USP standards for LVPs came after standards were first established in Australia and Britain. The Australian standards were based on the research by Vessey and Kendall (26) and 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 LVP 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

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

Particle size (≥μm)	Vessey and Kendall (26)	Australiaa (27)	British Pharmacopoeiab (28)	U.S. Pharmacopeiac (2)
2	1000	—	1000 (500)	—
3.5	250	—	—	—
5	100	100	100 (80)	—
10	25	—	—	50
20	—	2	—	—
25	—	—	—	5

aMean 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.

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 ml at particle sizes of 5 μm and 20 μm, respectively, are permitted in LVP 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. (29) 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 tested. Their proposal uses a term called statistic ST which is defined mathematically as

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

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

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

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

where S is the standard deviation. The advantage of the ST approach over the Australian draft standard is the increased stringency of the ST 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 ST were 101.3, the sample would pass the Australian test ($\bar{x} + 2s$ + 2s less than 200) but fail the ST test (value ≥ 100). The disadvantage of the ST 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. (29). The authors conclude by stating that an ST target of zero does not add unrealistic constraints on manufacturers, but it does reduce the risk of passing a lot of LVP solution having a few containers that are extremely contaminated with particles.

Groves (30,31) proposed that a single numerical value, the index of contamination (C), be adopted as a standard for accepting or rejecting LVP 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 at a size threshold of around 6.0 μm. Using the log-log relationship for the BP limits between 2 μm and 5 μm and extended the line to 6.0 μm, the extrapolated number of particles equals 63.244. From previous derivations outlined by Groves and Wana (31), the index of contamination may then be defined as

$$C = (\ln N1.0 - \ln 63.244)/m$$

where N1.0 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 the 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 later in this chapter). 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 will be reviewed in a later section of this chapter.

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 electronic 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.

The Task Group No. 3 of the Parenteral Drug Association published guidelines to be considered in the design and evaluation of visual inspection procedures (32). These guidelines will be 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 light bulb, 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 over that normally used. As light intensity begins to weaken, due to age 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.

Many manufacturers have progressed to the use of automated inspection equipment. Appropriate inspection procedure should specify and monitor the adjustment of the machine's operating parameters required to achieve a quality of inspection that is at least equivalent to that resulting from a previously established manual inspection procedure. Automated inspection machinery will be discussed in the following, 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 these 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 assures product quality and minimizes loss of expensive drug products.

Standard operating procedures for inspection of parenteral containers depend on the kind of container inspected, that is, procedures will be slightly different for ampuls 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, and an example of such a procedure is given in Table 3.4.

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 particulate 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. Reject any container having visible particles at any time during the inspection process.

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 acceptable 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 resulting 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 re-inspected by qualified inspectors to assure the quality of the inspection and the development of the trainee. After the inspector has passed his/her training period, performance tests should be done at random intervals to assure that quality standards are being maintained.

Two reports have been published concerning the effect of personnel experience on detection of particles in ampuls. Graham et al. (33) found that inspectors with no experience and inspectors having at least 10 years experience agreed 64 to 83% of the time with ampuls inspected under various conditions. Experienced inspectors were faster in the inspection process. Baldwin et al. (34) found that experienced inspectors reject ampuls at a greater rate (28.3%) than did non-experienced 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 product manufacturers as a very serious (critical) defect. Therefore, acceptable quality levels (AQL, the highest percentage of defective units acceptable for releasing the bath) for statistical sorting samples taken for particulate and other quality inspection generally is 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 re-sorting 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 who will inspect 50150 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 five 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 non-gridded 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 0.45μm filter. The maximum allowable number of particles for the entire membrane filter pad used as the blank control are:

10 μm 3

50 μm 1

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 40x 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 upon 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 purpose100% nondestructure 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 (35). 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, were comparable. Autoskan was not superior to manual inspection in terms of repeatedly rejecting those ampuls 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 of the same batch of ampul products. An example of rejection rates comparing machine and human inspectors is given in Table 3.5.

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%	19.729.2%
B	20.2%	21.5%	17.524.0%
C	20.3%	17.4%	15.519.5%
D	7.2%	7.9%	5.610.0%

Source: Ref.35.

Reproducibility in human visual inspection was the subject of a paper by Faesen (36). Each of 1000 diamond-numbered ampuls 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 ampuls only). The total number of rejects was registered following 60 inspections for the 1000 ampuls (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 those performed with the inspection cabinet. For ampuls the value reached using the Ampul Inspection Machine was less than 10% units higher 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 ampuls (3739), it was stated that the average reject rate for ampuls inspected manually was 22.5%. Three inspection machines were compared to manual inspection. Autoskan equipment (37) showed a reject rate of 14.1%, the Rota Machine (38) increased the reject rate 0.51.5% over that for manual inspection, and the Strunck Machine (39) yielded a reject rate of 2.98%. The major advantage of machine inspection was simply their substantial increase in the number of containers inspected per unit time.

Blanchard et al. (40) 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 methods 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 $\leq$ 40 μm. This, of course, presents some discomfort for those who believe that particulate matter, especially in the size range of 1040 μm, is clinically hazardous.

It is not only the size, but also, and probably more importantly, the number of large particles injected into man intravenously that is considered dangerous. Thus, official standards have been enforced for maximum allowable numbers of certain-sized 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 (7). Five milliliter ampuls containing 10 to 500 particles per ml of particle sizes between 5 and 40 μm (using polystyrene beads) were inspected by 17 inspectors in a standard booth. Based on a multiple linear analysis model that calculated the probability of rejecting an ampul 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

Table 3.6 Size of Particles of Varying Probability Levelsa

Particle concentration	Particle size (µm) 50% chance	Particle size (µm) 100% chance
USP limit 50 particles/mlb	18.82	51.45
USP limit 5 particles/mlc	19.96	54.88
1-ml ampul, 1 particle	20.07	55.21
2-ml ampul, 1 particle	20.08	55.25
5-ml ampul, 1 particle	20.09	55.28
10-ml ampul, 1 particle	20.10	55.29
20-ml ampul, 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

aArcsin $\sqrt{P_r}$ = 0.33689252 + 0.02231515 size + 0.000035 size vs. concentration -0.00008694 concentration.

bNot more than 50 particles/ml equal to or larger than 10 µm.

cNot more than 5 particles/ml equal to or larger than 10 µm.

Source: Ref. 7.

be qualified for in-line inspection. However, it is interesting to note that a minimum 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 analyses resides in the fact

that the tests are destructive. One hundred per cent inspection of each final container of parenteral product 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 electrooptic systems. Such systems are rapid, non-destructive, and reproducible in their inspection of parenteral products for foreign matter.

Early attempts to automate the 100% inspection process were reviewed by Groves (3). Systems developed and tested included the Brevetti device (41), Strunck machine (42), and the RCA machine (43). 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. (44) 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 particles of 10 μm 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 (45). 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. These systems will be discussed in the section Automated USP Particulate Matter Test on page 215. 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 AIM system, Seideneider and the Schering PDS/A-V system. The Prototron system (46,47) at one time was a widely used non-destructive inspection method using laser light, but is no longer used today.

Autoskan System

The Autoskan system uses white lightin contrast to laser light, which was used by the Prototron systemto 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. That product container will not be released by the Autoskan 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, ampuls, cartridges, and syringes. In Figure 3.1, ampuls on a rotary feed table are fed into the turret. The turret picks up the ampuls and intermittently transports them around to the inspection station where the lens of the television camera is located. The ampul is magnified by high-intensity light from below the check holding the ampul (see Figure 3.2). This light reflects particles moving in the liquid, making them visible to the camera (often visible also to the human eye). The Autoskan checks in the turret contain motors that spin each container 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 pre-set. 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.

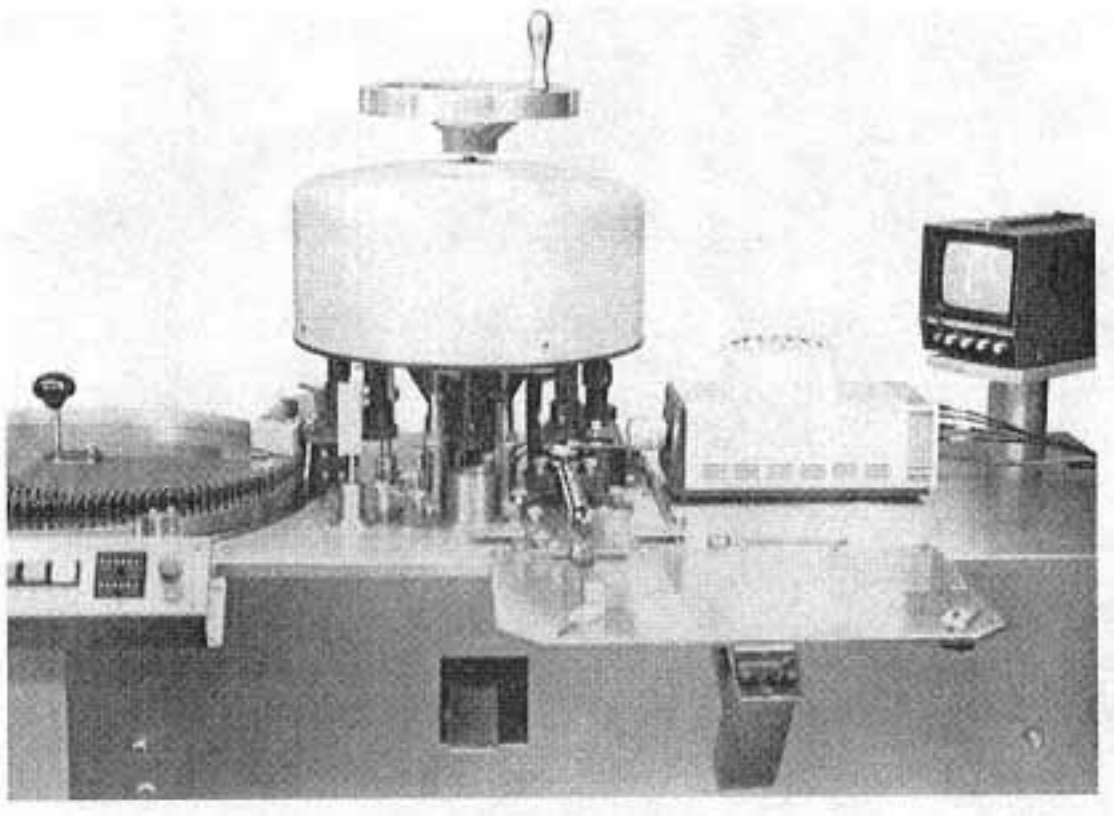

Fig. 3.1
Autoskan inspection system showing ampuls on a rotary feed table leading into and exiting from the detection area (courtesy of the Lasko Company, Leominster, Pennsylvania).

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 comparative 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 span for checking the liquid level, taking a master picture, and subsequent comparison pictures is less than one 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

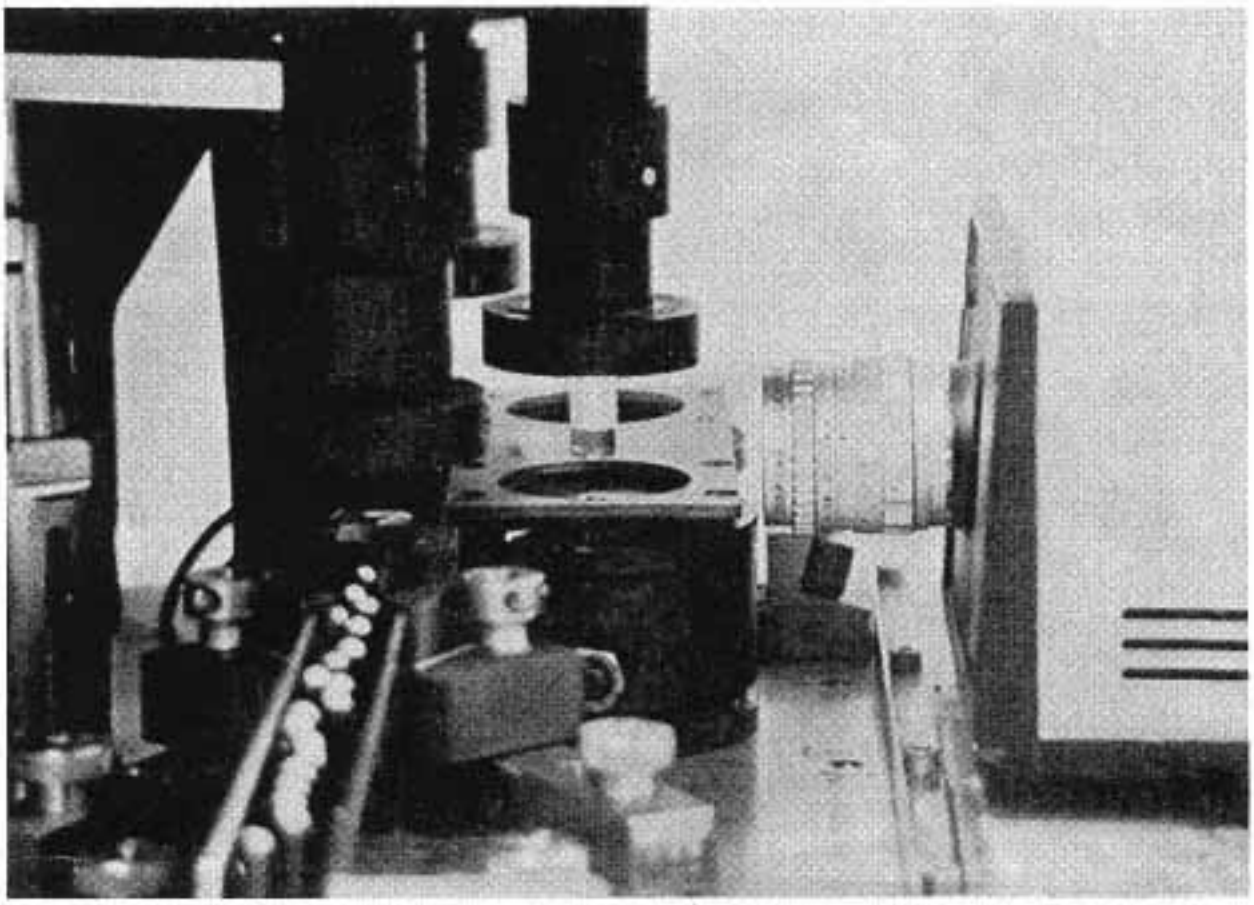

Fig. 3.2
Close-up of ampul being inspected for particles by the Autoskan system (courtesy of the Lasko Company, Leominster, Pennsylvania).

implication. The master picture is based on a motionless container. All scratches, printing, or other marks on either the outer or 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. (48) compared Autoskan's performance against visual inspection for discrimination of good and bad ampul 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 ampuls by the machine whereas the percentage for visual inspection was

Table 3.7 Comparison of Visual Inspection vs. Autoskan Automatic Inspection of Ampuls for Particulate Matter

	Visual inspection 50 examinations (avg.)	Machine inspection 10 runs
Ampuls (n = 50) rejected from the control bad lot	26.6	42.7 out of 46
% of bad lot rejected	53.2	92.8
Ampuls rejected from the control good lot	3.16	39.6 out of 752
% of good lot rejected	0.42	5.25

Source: Ref. 48.

only 54%. The rejection of good ampuls by visual inspection was significantly lower than the rejection of good ampuls by the machine.

Eisai Ampul Inspection Machine (AIM) System

Like the Autoskan system, the Eisai 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 pre-set 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 ampul 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 ampul sizes, ampul color, and viscosity of the liquid contents.

The conveyance and inspection mechanism of the Eisai system is shown in Figure 3.3. Ampuls are conveyed by the star wheel onto the inspection table, spun at a high speed, and stopped before reaching the light beam. When the ampul enters the light beam, the light projector and detector follow the ampul while liquid is still rotating inside. After one ampul is inspected by two sets of projectors and receptors (thus, a double inspection system) the next ampul is carried through the same process. Ampuls are moved on by the screw conveyor to the sorting pendulum, where rejected and accepted ampuls 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: Upjohn, Organon, and Merck Sharp & Dohme. The complete report of these evaluations is available from Eisai U.S.A., Inc. One evaluation was reported at the 1982 Annual Meeting of the Parenteral Drug Association. All three investigators concluded that the inspection of ampuls by the AIM system compared to manual inspection resulted in a great improvement in the quality of ampuls accepted and released. Using the performance criteria model published by J. Knapp et al. (to be 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 ampuls 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 the quality of those containers manually inspected. Advantages of the Eisai system itemized by the Merck report include

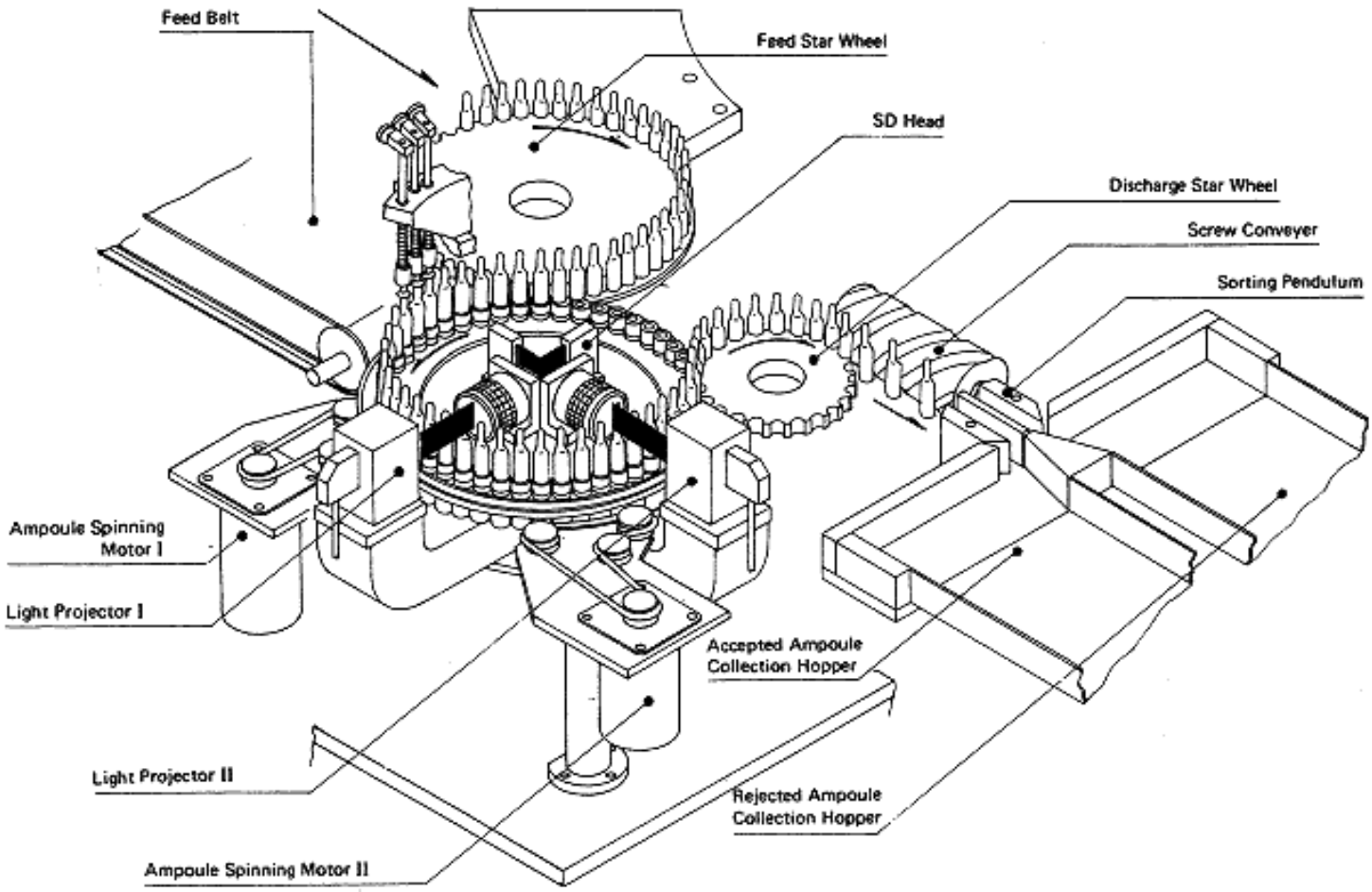

Figure. 3.3
Conveyance and inspection mechanism of the Eisai automatic inspection system (courtesy of Eisai, USA, Inc., Torrance, California).

(1) versatility, that is, ability to handle a large variety of ampul and vial sizes, products, and viscosities, (2) the adjustable sensitivity level, (3) attainable speeds, (4) results of performance studies, and (5) the price.

To this author's 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 (49). A photograph of the system is shown in Figure 3.4.

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; 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 the papers published by Knapp et al. (4953).

Probabilistic Particulate Detection Model

Knapp and his co-workers published a series of papers describing the theory and application of a probabilistic inspection model in the automated non-destructive particulate analysis of sealed parenteral containers. The probabilistic model is based on the finding that particulate

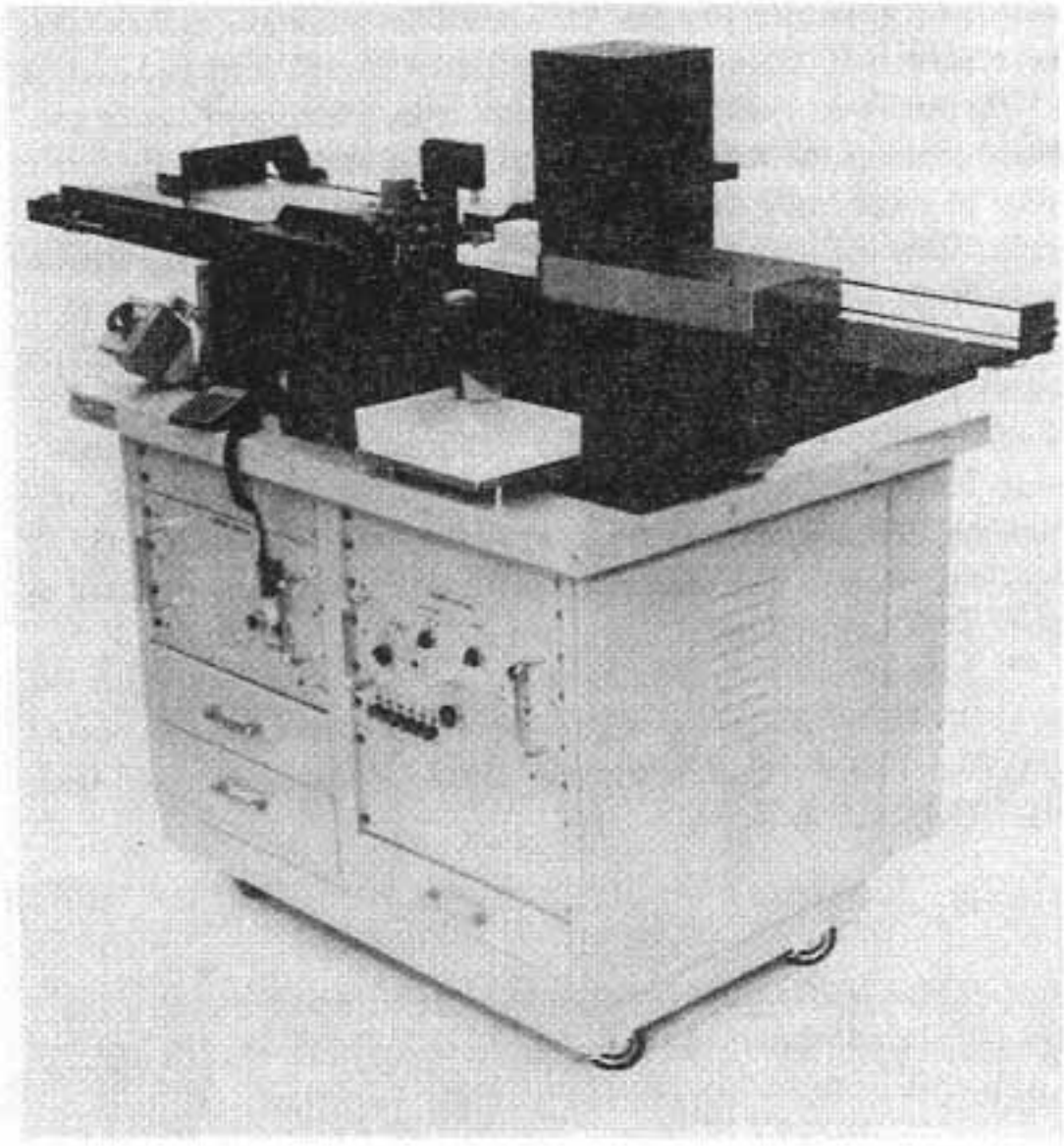

Fig. 3.4
Particulate Detection System 100 for ampuls and vials (courtesy of Electro-Nucleonics, Inc., Fairfield, New Jersey).

inspection methodologies, human or robotic, are probabilistic rather than deterministic in nature (50). 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 Figure 3.5 and represents the cornerstone for the conversion by Knapp et al. of particulate inspection from a craft to a science (53).

The abscissa in Figure 3.5 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 particulate-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 (50)

$$P(Mn)i = P(Ml)i$$

where P (Ml)i is the rejection probability associated with the nth manual inspection in a probability group, P (Ml)i is the quantity 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 Ml)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 (51). From the information contained in the reinspection histogram of Figure 3.5, Knapp and Kushner (50) defined three zones within the rejection probability limits of 0 and 1.

The accept zone contains all vials that have less than one chance in 10 of rejection in two sequential inspections. The reject zone contains all

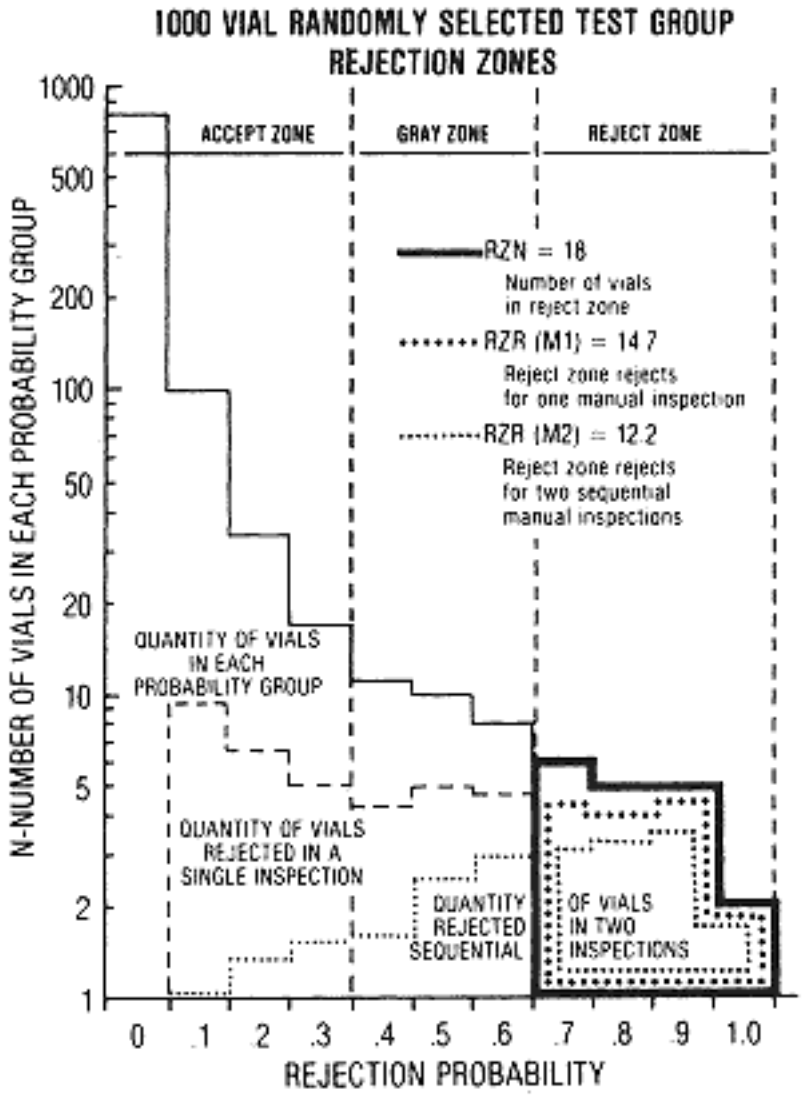

Fig. 3.5
Histogram plotting number of vials
per each probability of rejection group (courtesy of J. Z. Knapp, Schering Corporation, Kenilworth, New Jersey).

vials that have at least one chance in two 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 Figure 3.5 where

Accept Zone $p \leq 0.3$

Gray Zone $0.3 \leq p \leq 0.7$

Reject Zone $p \geq 0.7$

Figure 3.5 also shows three terms abbreviated RZN, RZR (M1) and RZR (M2). The definitions of these terms are given in the figure. Their calculations are explained thoroughly in Reference 50. 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 Figure 3.5, 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 GMP requirement for validation of any alternative inspection technique or process (50).

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 (49,51). 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 (54). 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.

USP Test for Particulate Matter in Large-Volume Injections for Single-Dose Infusion*

After several years of collaborative effort among laboratories from the FDA, universities, and pharmaceutical manufacturers, a method became

*The March-April, 1993 issue of the *Pharmacopeial Forum* (pp. 49394956) contains proposed revisions, many of which are significant, in the USP XXII section (Chapter <788>) on Particulate Matter in Injections. These will be discussed in Appendix VI at the end of this book.

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). The method involves the filtration of 25 mls of solution through an ultraclean membrane filtration assembly, then observing the membrane and counting entrapped particles on its surface under a microscope using 100x magnification. Analysis by microscopic techniques suffers from several disadvantagesit is very time-consuming, requires technical expertise, and, because of the manpower requirements, can be very expensive. Several alternatives to the approved USP method have been accepted for particle counting, most recently the HIAC electronic particle counter. However, if any dispute arises regarding fulfillment of USP particulate matter specifications, such disputes must be settled by applying the official USP microscopic method.

General

The present USP method provides both qualitative and quantitative data on particulate content in LVP 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 (LAF) Hood

All operations and manipulations must be performed under a certified laminar flow hood equipped with ultra HEPA (high efficiency particulate air) filters. Air flow velocity must range between 70 and 110

feet per minute as measured with a calibrated velometer. Laminar flow hood certification has been 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. 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 solvents used in the filtration process.

Introduction and Use of Equipment in the LAF 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 (1) warm detergent solution, (2) hot water, (3) water, and (4) 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.

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 handing 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 that a color contrast grid membrane filter be used, but does not specify the porosity of the membrane. The porosity must be sufficiently small to entrap all particles ≥ 10 μm. Most laboratories use cellulosic type membranes in the porosity range of 0.81.2μm.

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. Pressures 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 (55) 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 mls are transferred to the filtering funnel. After standing for one minute, a vacuum is applied to filter the 25 ml sample. Filtered water is then applied with the vacuum off 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 10x objective, and have one ocular equipped with a micrometer able to measure accurately particles of 10 μm and 25 μm linear dimension. Incident light should be

set at an angle of 10 to 20 degrees, although an angle of 30 to 35 degrees has been reported to be more effective in illuminating the membrane surface inside a plastic Petri slide (56). Calibration of microscope micrometers based on a National Bureau of Standards primary standard stage micrometer has been described by Lanier et al. (57).

Particles are counted under 100x magnification with the incident light at an angle of 10 to 20 degrees. Obviously, this is a slow and tedious process requiring patience and dedication on the part of the microscopist. Use of higher magnification, up to 400x, may be necessary occasionally to discern discrete particles from agglomerates or amorphous masses (56). Sometimes particles not visible with dark field reflected light are very easily observed by means of bright field illumination at 45° polarization.

Two sizes of particles are counted, those having effective linear dimensions ≥10 μm and ≥25 μm. 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. Counts from the blank membrane are subtracted from the sample membrane counts. Blank membrane counts rarely are zero. However, if 5 or more particles ≥ 25 μm 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.

The USP specifically requires all test preparations and blanks to be performed in duplicate. Following the subtraction of blank counts from sample counts and averaging the results, should the net particle counts exceed the limits specified by the USP (not more than 50 particles per ml ≥40 μm and not more than 5 particles per ml ≥25 μm) the large-volume injection product fails the USP test for particulate matter.

LVP particulate matter standards in other countries governed by other compendia will be reviewed in a section at the end of this chapter.

Particulate Matter in Small-Volume Parenterals*

It has been generally accepted that the particle load in large-volume parenteral (LVP) solutions has been substantially reduced because of the USP particle limits placed on these solutions back in 1975 (25). It has also been a general consensus that these LVP limits are too strict for small-volume parenteral (SVP) solutions and, in fact, SVPs should not have particle limits because (a) volumes administered are much smaller than those for LVPs, and (b) health hazards from injected particulates have not been unequivocally established. Nevertheless, the USP sponsored studies to establish particle limits for SVP solutions reasonable from both a safety standpoint and a quality control standpoint achievable by the parenteral industry.

Two SVP particle limit proposals were published in late 1983 (58). One based on particles per container was proposed by the USP Subcommittee on Parenteral Products; the other proposal, by the USP Panel of Sterile Products, was based on particles per ml. 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 ml	250 Particles per ml

The subcommittee proposed limits based on the following rationale: the addition of up to five containers of any SVP to a 1 liter LVP solution should not increase the number of particles by more than double those allowed by the USP limit for LVP solutions (five particles per ml ≥25 µm or 5000 particles in 1 liter ≥25 µm; 50 particles per ml ≥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 LVP containing 5000 particles ≥25 µm, the total

*See footnote on p. 206.

particles ≥25 µm 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 LVP would be 100,000, which would be no more than double that of the LVP alone. Therefore the subcommittee proposal was based upon concern more for the cumulative particulate insult the patient might receive than for the number of particles per ml 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). 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.

Of the more than 500 official SVP products in the USP, 134 of these products (58) 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 anti-cancer therapy, or for episodic use are excluded.

While many laboratories prefer to employ the LVP microscopic method for counting particles in SVP 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. 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 which is available from only one major U.S. supplier.

Other concerns over the proposed USP test for SVP particulate matter included lack of a sufficient data base 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 in specific details in the calibration, preparation and determination sections of the test, and the lack of consistency between the LVP and SVP tests for particulate matter. These will be discussed in more detail. The USP (Section 788) requirement for particulate matter in small-volume injections (SVI) became effective in 1986. The test calls 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 recommends Pacific Scientific, makers of HIAC/ROYCO particles counters and using sensors manufactured by Russell Laboratories.

All SVI products containing 100 mls or less are required to pass the USP test. This includes reconstituted solutions. Products exempted from the USP test are prefilled syringes and cartridges unless an individual monograph states specifically that a product in such delivery systems is to be tested. The requirement also does not apply where the monograph specifies that the label shall state 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 resolutionuse monosized 10µm particle standards to assure 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 ratecertify that the actual flow rate is within the manufacturer's specification for the particular sensor used.

3. Sample volume accuracysince 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.

Automated USP 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 (59), 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., 510 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 upon 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 that were described in the section Visual Inspection: Automatic Methods. Image analysis is not a 100% final container 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 (59). According to 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, where 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 hard copy record, and stored on magnetic discs 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 (60). The πMC system consists of (1) a microscope and television camera that illuminate and observe the sample on the filter membrane, (2) 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 (3) 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 in review are:

1. They conform to USP procedure for particle analysis of large-volume injectable solutions.

2. Particles are counted, sized, and shape-characterized with much greater speed and precision as 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 (61) and have suggested new methods such as automated microscopy (62).

Recent 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) (63).

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 nanometer and 1 micrometer 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 micrometers and larger) in solutions can be measured. This technique also permitted three-dimensional shape-mapping of particles and holds considerable promise for non-destructive 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 chromatography. The result is a high-efficiency separation of a particle mixture according to weight. Depending on the experimental conditions, field-flow fractionation can separate particles in the 0.011 micrometer or 1100 micrometer ranges.

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 (64). 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 (65). 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 than microscopic methods. These advantages and disadvantages will be 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) (6668)

The Coulter Counter (Figure 3.6) detects particles by measuring the change in electrical resistance produced when 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 (68). 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 micron 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

Fig. 3.6
Photograph of Coulter Counter Model ZM (courtesy of Coulter Electronics, Inc., Hialeah, Florida).

proportionality of resistance change and particle volume to be valid (69). 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 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 I.V. solution. Counts measured in this extremely small volume may be too low (1020 particles) for useful statistical accuracy of sampling (70). 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 (3).

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 (71). 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 accurately 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 intra-venous solutions can be detected easily and rapidly by the Coulter Counter (69, 72-76). 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 I.V. 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 Figure 3.7. A tungsten lamp produces a constant collimated beam of light which 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.

HIAC (High Accuracy Instruments Division) particle counters employ the light-blockage principle in the detection and quantitation of particulate matter in parenteral solutions (see Figure 3.8). These instruments count approximately 4000 particles per second. HIAC counters

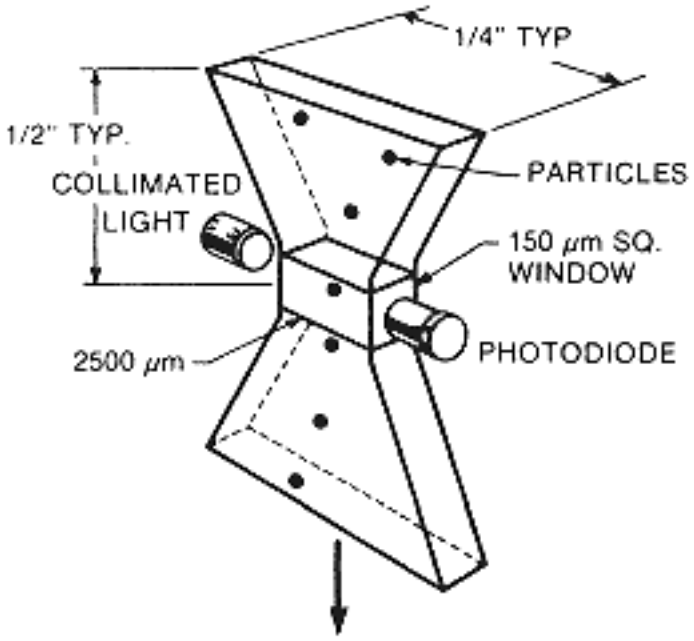

Fig. 3.7
Schematic representation of the light obscuration principle (courtesy of HIAC/Royco, Menlo Park, California).

use sensors having size measurement ratios of 1:60. In other words, a 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 150 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. Advantages for using HIAC particle counters have outweighed the disadvantages. Lantz et al. (77) 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 were as follows:

Advantages

1. Particles are counted automatically.

2. Parenteral solutions, either electrolytes or non-electrolytes, could be counted.

Fig. 3.8
HIAC/Royco's System 8003 for parenteral particle counting to comply with the requirements of USP <788> (courtesy of HIAC/Royco Division of Pacific Scientific Company, Silver Spring, Maryland).

3. The instrument was easy to calibrate and use.

4. Replication of counts was good.

5. Ability to vary the volume of samples 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 as 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 Figure 3.9.

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 which then focus the light onto a photo-detector. The light trap seen in Figure 3.9 is designed to absorb most of the main light beam photons.

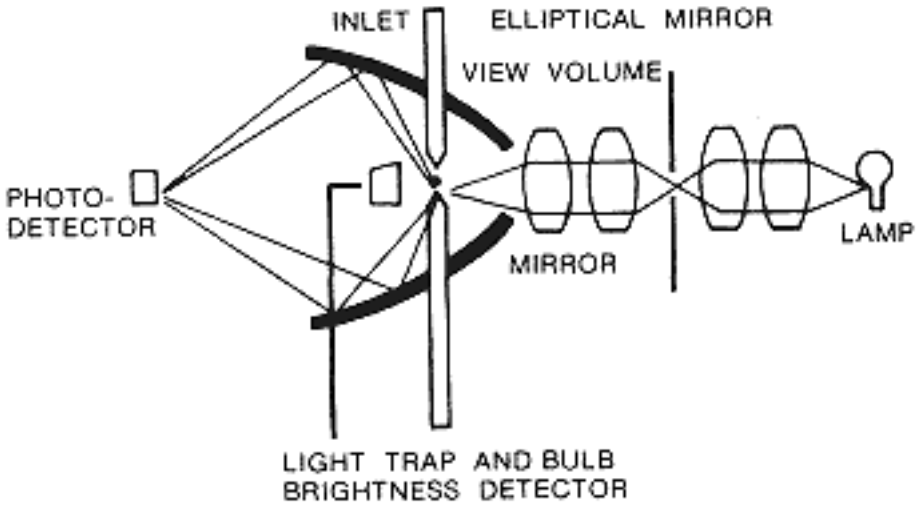

Fig. 3.9
Schematic representation of the light-scattering principle (courtesy of Climet Instruments Co., Redlands, California).

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 liquidborne 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 100 ml per minute samples rates, 3,500 counts per ml, and simultaneous measurement of six particle sizes.

Davies and Smart (78) reported on rapid assessment of particle levels in small-volume ampul products with good reproducibility, using the scattered-light based particle counter. Advantages 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.

Comparison of Microscopic and Electronic Particle-counting Methods

The comparisons discussed in this section will 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 particles as a three-dimensional object and measures the volume consumed by the particle. Thus, the microscope, HIAC, and the Coulter Counter methods size particles in one, two, and three dimensions, respectively.

Table 3.8 Commercially Available Particulate Measurement Systems for Parenteral Use (79)

Type of System	Model Evaluated by the FDAa	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	Recommended 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

aOxborrow, GS, A comparison of particle counters from five manufacturers, presented at the *May, 1987 Meeting on Liquid Borne Particle Inspection and Metrology*, Washington, DC.

An excellent theoretical discussion by Schroeder and DeLuca (71) showed that it is virtually impossible to correlate instrumental and microscopic particle counts directly for irregularly 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 × 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 that the size-count relationship follows the conventional log-log relationship, the theoretical HIAC count of 50 ellipsoid particles of 10 µm size 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/ml at 10 µm and 5 particles/ml at 25 µm. Assuming the HIAC method to follow 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 on 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, the size correction factor of the Coulter Counter for ellipsoid-shaped particles is 0.52. Applying the same log-log relationship for a 50 particle/ml sample, the Coulter Counter will yield a count of only 9.7 particles of size 10 µm or larger.

Hopkins and Young 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

Table 3.9 Summary of Sphericity Correction Factors Based on Longest Linear Dimension

Shape	DO Longest dimension	DH Horizontal projection	DA Light blockage	DV 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 dia.)	1.00	0.81	0.62	0.52
Fiber (rigid, 10:1)	1.00	0.64	0.36	0.25

Source: Ref. 71.

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 microscope. 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/ml $\geq$5 μm) 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.

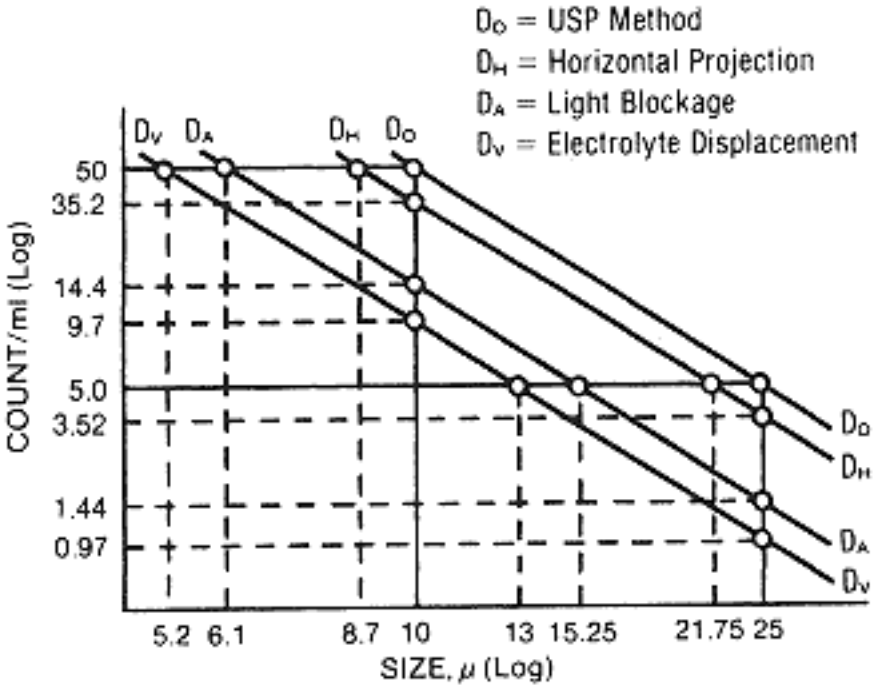

Fig. 3.10
Log count vs. log size corrections for sizing and counting prolate ellipsoids (from Ref. 71).

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/ml	50		17.30	49.16

Source: Ref. 71.

Table 3.11 Comparison of Microscope, Coulter, and HIAC Data, Showing Total Counts of Particles Greater than 5 µm

Sample	Microscope	Coulter	HIAC
5606A, Supplier A	6,285	6,867	5,080
5606A, Supplier B	19,364	23,534	18,000
5606A, Supplier B	5,113	7,200	5,020
5606A, Supplier A	4,285	4,635	3,660
5606A, Supplier A	6,675	8,715	6,930

Source: Ref. 80. Data from Technical Documentary Report No. ML-TDR-6472, Air Force Materials Laboratory, Wright-Patterson Air Force Base, Ohio.

A similar conclusion was reached by Rebagay et al. (81) 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

Table 3.12 Particles per ml of Isotonic Saline Solutions in the 550 µm Size Range

		HIAC	Coulter	Microscope
MFG A S-1a	Average	10.8	17.4	11.9
MFG B S-2a	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
Filtered water				
blank	Average	0.9		

aAir bubbles in solution.

Source: Ref. 80.

with particles that possess morphological and optical characteristics similar to the particles of interest. An example of their data measuring particle content of various intravenous infusion solutions is given in Table 3.13.

Lim et al. (82) filtered various small-volume parenteral solutions and counted particles using the manual counting method under the microscope and the electronic Millipore MC method. In products with relatively few particles, both methods gave similar results. In products containing a high number of particles in the size range of 525 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. (83) in comparing the microscope and the Prototron laser beam (using the light-scattering principle). With solutions containing abundant particles of the small-sized range, the particle counter gave more reliable and accurate results than did the microscope.

Current Issues with Electronic Particle Counters

Knapp and DeLuca (79) listed problems encountered to some degree with all available instrumentation used to measure particulate matter (Table 3.8). Many of these problems were also discussed by Knapp (84) where he also proposed action steps to overcome these problems. Today, many of these problems have been resolved with advances in the instrumentation available.

Barber (85) published an excellent paper detailing the limitations of light-obscuration (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 on Principle of Light Obscuration (pp. 221224) 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

Table 3.13 Particulate Matter Determination of Some Intravenous Solutions by Automatic and Microscopic Methods

	Microscopic methoda			Automaticc	
Infusion solutions	I	II	Image analyzerb	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

aReichert Zetopan Universal Microscope. I, Incident polarized light and polycarbonate as substrate; II, incident bright field lighting and cleared white cellulosic substrate.

bTIMC computer measurement method with cleared white cellulosic substrates.

cHIAC counter, calibrated with I, AC Fine Test Dust, II, polystyrene-divinylbenzene spheres.

Source: Ref. 81.

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 (>÷µm), and spherical, good numerical accuracy is possible. However, when particles are many, small (≤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, and 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 rates pulses may not rise to full height, resulting in undersizing (85).

Nonspherically-shaped particles produce significant errors in 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 increases, 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 monosized spherical latex particles which provide a very narrow range of known monoshaped 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 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 ($\leq$1 μm) microdroplets. Only in significant numbers do silicone microdroplets produce significant errors in particle measurement. Air bubbles are also problematic, but the USP XXII provides for a method of degassing the sample using ultrasonification. Such degassing does not remove all microscopic bubbles nor reduce the dissolved air content in the solution.

Sampling variability, as with any quality-control test relying 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) (86). The USP had a three stage proposal: (1) an increase in the minimum volume

Table 3.14 Problems Encountered with Electronic Particle Counting Systems(79)

Low sample volume handling capacityimposes sampling errors due to loss of particulates during sampling manipulation before and during analysis
Shape-dependent signals
Inadequate particle size range
Specification of inappropriate measurement limitsnumber 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

of sample to be tested, (2) an increase in the minimum number of test units to be testing, and (3) 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: (1) provide data and information regarding the possibility of tightening the light obscuration limits in the USP, (2) seek and provide silicone assays that could be used for testing at Stage 2, and (3) study the adoption of 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 LVPs the USP relies on

membrane filtration in 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 inaccurate particle test results regardless of the test used. Ernerot (87) 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. (56) 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 LVP 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 upon the particles and affect the reproducibility of the test results.

Temperatures affects the number of particles found in parenteral solutions. As shown in Figure 3.11 (88), 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

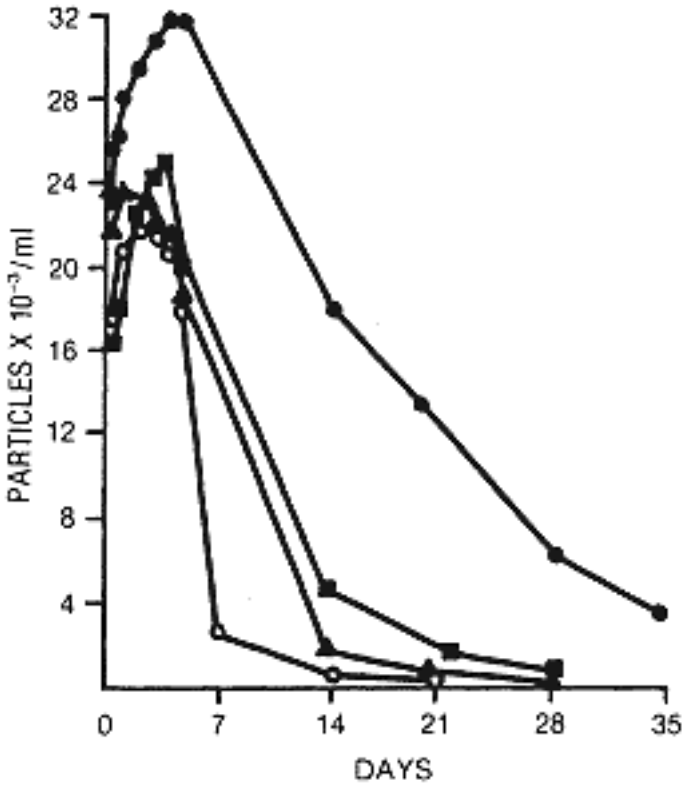

Fig. 3.11
Number of particles between 2.33
and 5.02 µr found in resting plastic bags after shaking for 30 hours
as a function of time and
temperature. Key: ■, 35°C; ○, 45°C;
▲, 55°C; ●, Room temperature (from Ref. 88).

these smaller diameters but an increase in particle counts at 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 was constructed based on a paper by Lotteau (89) and a review of the available compendia. Review of Table 3.15 brings out some interesting comparisons:

Table 3.15 Comparison of Compendia for Particulate Matter Standards(89)

Compendia	LVP/SVP	Method	Limits
USP	LVP	Microscopic	≤50 part/ml ≥10 µm
			≤ 5 part/ml ≥25 µm
	SVP	Light obscur.	≤10,000 part/container ≥10 µm
			≤ 1,000 part/container ≥25 µm
BP	LVP	Coulter counter	≤ 1,000 part/ml ≥2 µm
		light obscur. is	≤ 100 part/ml ≥5 µm
		proposed	
Italian	LVP	Light obscur.	≤100 part/ml ≥5 µm
			≤ 4 part/ml ≥20 µm
French	LVP	Microscopic	None
		light obscur.	
		for quant.	
European	LVP	Being drafted	Being determined
Japan	LVP	Microscopic	≤20 part/ml ≥10 µm
			≤ 2 part/ml ≥25 µm
	SVP	Light obscur.	≤1000 part/container ≥10 µm
			(proposed)

1. Only the United States (USP XXII) contains a requirement for particulate content in small-volume parenteral products

2. Only the British Pharmacopeia designates the use of the Coulter counter as the electronic method for measuring particulate matter in large-volume solutions

3. 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

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Introduction

Package integrity is a measure of a package's ability to keep the product *in* and to keep potential contaminants *out*. While this is a simple concept, it is not so simply measured or validated. The product necessitating containment consists of the liquid or solid parenteral drug product, as well as the gas headspace in the case of a package sealed under vacuum or with an inert gas. Potential contaminants include microorganisms, pyrogens, other chemicals or materials, and particulates. Various tests are used by the pharmaceutical industry to measure parenteral product package integrity. However, many of these tests are insensitive and qualitative. Validation of package integrity is rarely determined by evaluating packages representing the full range of package component or production assembly variables. Only sterility of the packaged product is required by the Food and Drug Administration

(FDA) as verification of package integrity. Yet package integrity is a critical requirement of all parenteral products.

The apparent lack of advancement in parenteral package integrity validation and test method development may be due to industry's success at providing adequate sterile packaging. However, novel packaging designs and the need to minimize moisture pickup or gas headspace loss for very sensitive products challenge the traditional standards of integrity.

Fortunately there is a large body of knowledge on the subject of leakage scattered throughout the literature belonging to other scientific disciplines. By gleaning a general understanding of leakage concepts, it may be possible to more logically design, assemble, and validate integral parenteral product packaging.

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) where 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. For permeation to occur, the molecule must be adsorbed onto the barrier then move through the material by dissociation and migration, then 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:

$$J = -D(\partial C/\partial x)_t \qquad [1]$$

where

J = amount of diffusant, g/m2.s

D = diffusion constant, m2/s

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 where (1,2):

$$\partial C/\partial t = D(\partial^2 C/\partial x^2) \qquad [2]$$

A graphic representation of permeation flux with respect to time is given in Fig. 4.1 (1).

Leakage, on the other hand, is a sum of convection and diffusion. Solubility of the diffusant in the barrier material plays no part in either the convection or 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

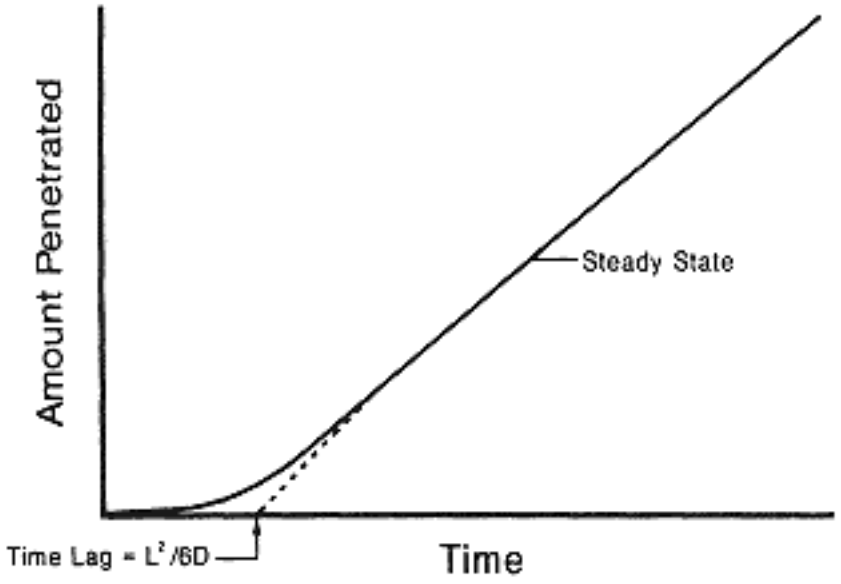


Fig. 4.1
Permeation flux vs. time where the time lag for the penetration of the diffusant is a function of the barrier thickness (L) and the diffusion constant (D) (courtesy of the Parenteral Drug Association, Inc., Bethesda, Maryland). Source: Ref. 1.

leakage gap. This relationship is described in Eq. 3, where the total flux for species A is a sum of the convective fluxes for all species, and of the diffusion of A which depends on the concentration gradient of only species A (3):

$JA = XA(jA + jB) - (p)(DA)(XA)(1/x)$ [3]

where

JA = total leakage flux of species A, g/m2.s

XA = fractional amount of species A, dimensionless

j = convective flux of each species, A and B, g/m2.s

p = density of mixture, g/m3

DA = diffusion coefficient of species A, m2/s

x = barrier thickness, m

This can be practically understood by considering a sealed parenteral vial containing a nitrogen headspace, which is 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 is about 6 psi less than inside the vial. 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 tends to convectively force nitrogen and liquid product out.

Leakage theory generally assumes a single component system where only convective flux occurs. In other words, only a total pressure gradient exists and there is no concentration gradient of gas across the seal. Examples of parenteral packaging situations that may require the consideration of diffusional leakage include an inert gas flushed vial where diffusional leakage across the seal could result in the loss of headspace integrity, or a wet-dry syringe where a rise in moisture level of the lyophilized cake could occur due to water vapor diffusional leakage from the wet chamber into the dry cake.

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 or temperature and pressure. For example, a carbonated beverage can may leak 10 cc of carbon dioxide in three months at 60 psig, or a submerged package may leak two bubbles per second of 1/8 inch diameter when pressurized to 40 psig (4).

The units of measure commonly used in many literature references to specify leakage rate are standard cubic centimeters per second (std cc/s). According to the international metric system of units (SI nomenclature) leakage is measured in pascal cubic meters per second (Pa.m3/s). In both expressions, units of gas mass (std cc and Pa.m3) 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, however small changes due to temperature variation are insignificant compared to the large differences in gas pressure or leakage quantity. All leakage rates given in this chapter will be presented in the more current SI units. To convert to std cc/s from Pa.m3/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 (5).

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, 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 10-3 Pa.m3/s (2,6). Laminar flow occurs for capillaries about 10-4 cm in diameter and measured leakage rates are approximately 10-2 to 10-7 Pa.m3/s (2,3,6,7,8). Laminar flow equations also apply to the movement of liquids through capillaries. Consequently, these equations have been used to calculate the amount of time required to detect leakage in ampules using the blue dye test (9).

Table 4.1 Leakage Units of Measure (5)

Pa.m3/sa	Std cm3/sb	Std L/dayb	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.m3/s

1 microcubic foot per hour (μft3/hr) = 1.0 × 10-6 Pa.m3/s

1 torr liter per second (torr L/s) = 0.133 Pa.m3/s

aPreferred SI nomenclature

bExpressed as quantity of gas (air) in a unit of volume at sea level atmospheric pressure

Molecular flow is most probable with leakage rates below 10-6 Pa.m3/s (2,6). 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 Equations 4 through 6, and are illustrated in Fig. 4.2. More detailed equations can be found in the references cited above.

$$\text{Molecular:} \quad Q = f(P_1 - P_2) \qquad \text{most probably} < 10^{-6}\ \text{Pa}\cdot\text{m}^3/\text{s} \qquad [4]$$

$$\text{Laminar:} \quad Q = f(P_1^2 - P_2^2) \qquad \text{most probably } 10^{-2} \text{ to } 10^{-7}\ \text{Pa}\cdot\text{m}^3/\text{s} \qquad [5]$$

$$\text{Turbulent:} \quad Q = f(P_1^2 - P_2^2)^{1/2} \qquad \text{most probably} > 10^{-3}\ \text{Pa}\cdot\text{m}^3/\text{s} \qquad [6]$$

where

Q = convective flux (mass/time)

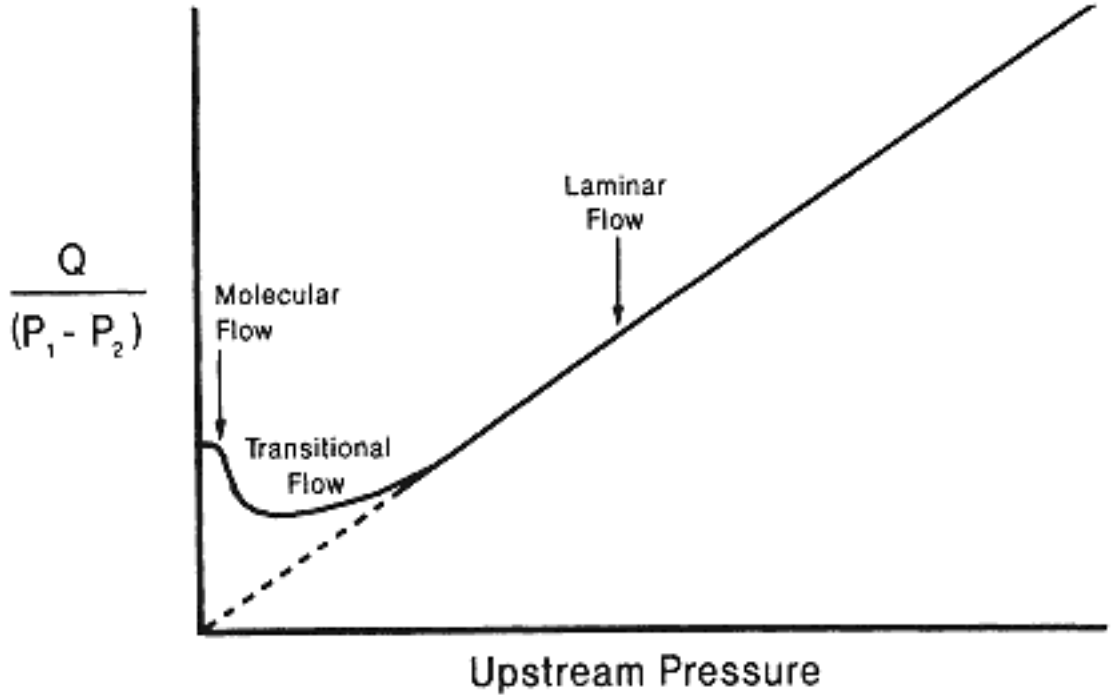


Fig. 4.2 Leak rate convective flux (courtesy of the Parenteral Drug Association, Inc., Bethesda, Maryland). Source: Ref. 2.

P1 = upstream pressure

P2 = 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 pick-up 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 10-10 Pa.m3/s. Vacuum vessels meet a leak rate specification of no greater than 10-6 Pa.m3/s (4).

Figure 4.3 illustrates approximate gas leakage rates and 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 substance that must be contained in the

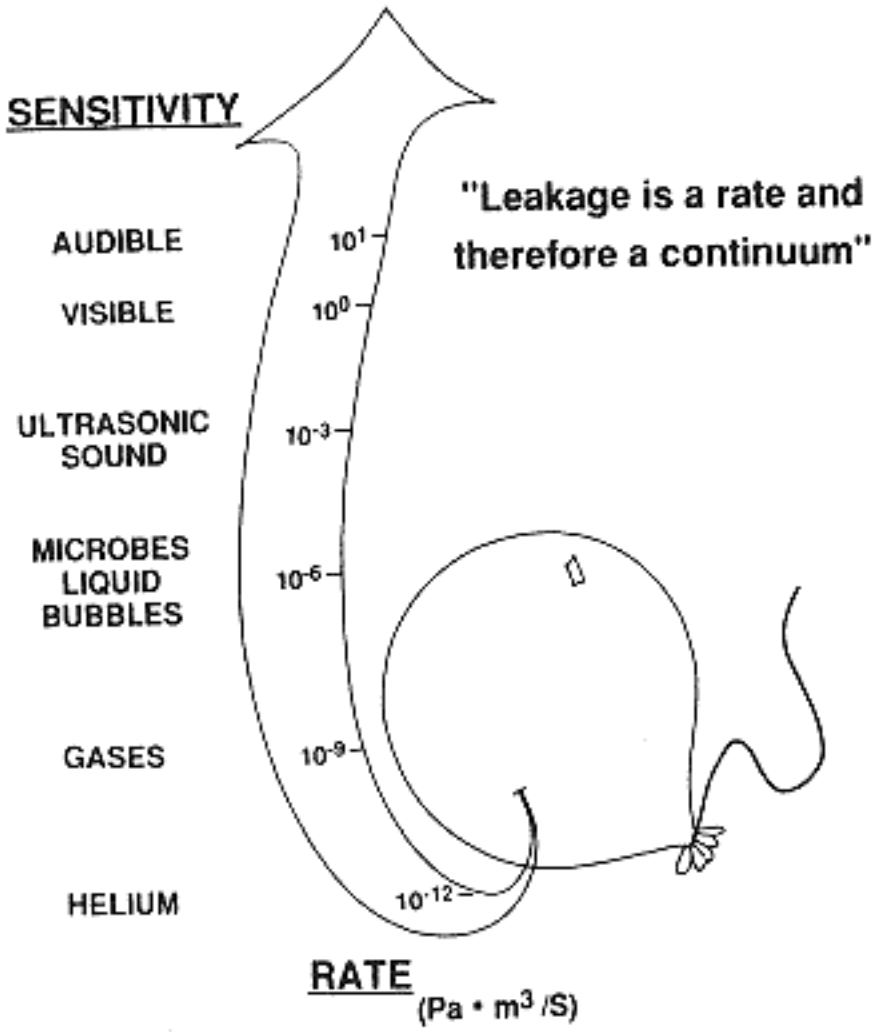


Fig. 4.3
Leak rate as a continuum.

package. Very rapid leakage is audible, as a whistling teapot for instance. As leaks get smaller they become imperceptible to the human senses. The smallest leaks to allow the passage of microorganisms and liquids are about 10-5 Pa.m3/s. Most gases cannot pass through leaks smaller than about 10-9 Pa.m3/s. Only helium gas can flow through the smallest leaks and is therefore useful for detection of leaks as small as 10-12 Pa.m3/s.

Expressions such as leak free, without leaks, or leak tight are meaningless by themselves. All packages leak to some degree. What is

meant by these 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

When setting acceptance limits for leakage, first determine what rate is required to assure acceptable package integrity. To avoid confusion, the leakage rate should be defined according to clearly stated test conditions of time, pressure, and temperature. The set of standard conditions most commonly accepted is that of dry air at 25°C for a pressure differential between one 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. When test conditions are not specified these standard conditions are generally assumed (5).

Let us consider the case of parenteral packages such as vials or ampules. Such packages must minimally keep out microbial contamination and at the same time keep the product from leaking out of the package. It has been shown that no visible water will leak when dry air, at the same pressure, leaks at a rate as great as 10-5 Pa.m3/s (10). If a leak is so small that liquids cannot pass, then microorganisms will also not be able to move through (11). Given 10-5 Pa.m3/s as the maximum allowable leak rate for a microbially sound seal, then a safety factor of 0.1 is usually recommended, and the leak rate specification is established as 10-6 Pa.m3/s (10).

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 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 two-year expiry. Knowing the headspace volume in the vial is 5 cm3, the maximum allowable leak rate can be 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 psia = 34,470 Pascals

5 cm3 = 5 × 10-6 m3

2 years = 6.3 × 107 seconds

Therefore, the maximum allowable leak rate is

(3.45 × 104 Pa) (5 × 10-6 m3) / (6.3 × 107 s)

or, 2.7 × 10-9 Pa.m3/s

Incorporating the safety factor rule of 0.1, the leak rate specification would be established as 10-10 Pa.m3/s.

In these examples the maximum acceptable leakage rate is defined according to SI units of Pa.m3/s. Such quantitative specifications are rarely established by the pharmaceutical industry, but are common place in the electronics, vacuum, nuclear waste, and aeronautics industries, for example. Alternatively, leakage can also be defined according to a more practical end point. A lyophilized product, for example, 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 grams, then the maximum allowable leak rate can be calculated as:

Leak rate = (mass of water pick-up)/expiry dating

= (4.0% - 0.1%) (2.0 grams) / 2 years

= 39 mg of water per year

From this, a reasonable safety factor would need to be incorporated to establish a leak rate specification defined according to recommended temperature/relative humidity storage conditions.

Leak Test Methods

Selection of Equipment

Once a leak rate specification has been defined, a leak test method must be selected that is capable of measuring such leakage rates. The leak test

instrument or method should be more sensitive by at least a factor of 2 than the minimum leakage to be detected in order 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 10-4 Pa.m3 is negligible compared with that having a sensitivity of 10-13 Pa.m3, which may run 10,000 times higher (5).

Leak test equipment generally have an optimum window of performance. For instance, bubble testing by immersion in water can accurately detect leakage rates of 10-2 to 10-5 Pa.m3/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 10-5 Pa.m3 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 10-2 Pa.m3/s may require steps to ensure that the package does not experience too rapid a pressure loss before the package can be immersed (5).

There are other considerations in the selection of leak test equipment including cost, ease of use, repeatability, testing speed, safety, and data storage, analysis, and display capabilities. Selection of leak test methods and equipment should be carefully catered to the package requirements and the needs of the user.

Methods

A summary of leak test methods, their levels of sensitivity, advantages and disadvantages, reported usage, and equipment suppliers is presented in Table 4.2. A few additional comments on these methods are presented below. More detailed information can be found in References 2, and 12 through 24.

Visual Inspection.

Visual inspection is certainly the easiest leak test method to perform, but, conversely, it is also the least sensitive. Visual inspection is one of

Table 4.2 Leak Test Methods

VISUAL INSPECTION

Method: Look for leaks

Sensitivity: 10-2 Pa.m3/s

Pros:	Cons:
Simple	Insensitive
Inexpensive	Operator dependent
	Qualitative

Reported Usage:

Used in combination with pressure and/or temperature cycling to accelerate leakage, improve sensitivity.

Suppliers:

Seidenader Inspection System, Morristown, NJ

Automated on-line visual inspection systems

BUBBLE TEST

Method: Submerge package in liquid, pressurize or pull vaccum, observe for bubbles. Apply surfactant and look for foaming.

Sensitivity: ≥ 10+6 Pa.m3/s

Pros:	Cons:
Simple	Relatively insensitive
Inexpensive	Operator dependent
Location of leaks can be observed	Wets package seal
	Qualitative

Reported Usage:

Pipes, large equipment, aerosol warm water bath test

Suppliers:

ARO Non-Porous Package Tester, Buffalo, NY

PRESSURE/VACUUM DECAY

Method: Change in pressure or vacuum is measured inside the package or outside in a sealed package chamber.

Sensitivity: ≥ 10-7 Pa.m3/s

Pros:	Cons:
Relevant to package performance (vacuum headspace; terminal autoclaving)	Sensitive to temperature, atmospheric pressure
Quantitative (depending on equipment)	
Clean, nondestructive	

(table continued on next page)

Table 4.2 (Continued)

Reported Usage:

Parenteral vials (vacuum headspace retention), food cans, flexible lid containers, screw-cap bottles, steel drums

Suppliers:

Shibuya International, Inc., Medessa, CA

Wilco Precision Testers, Tuckahoe, NY

ARO Leak Detectors, Buffalo, NY

MKS Systems, Inc., Andover, MA

Ion Track Instruments, Inc., Burlington, MA

S. Himmelstein and Co., Hoffman Estates, IL

DYE TESTS

Method: Movement of dye across a seal is visually or instrumentally detected.

Sensitivity: ≥ 10-5 Pa.m3/s

Pros:	Cons:
Widely accepted in industry	Qualitative
Easy	Destructive
Inexpensive	Slow

No special equipment required for visual dye detection

Reported Usage:

Ampules, vials, IV screw capped bottles, laminate film heat seals

Suppliers:

None

CHEMICAL TRACER TESTS

Method: Solution containing a tracer chemical is applied to one side of package seal. Pressure or vacuum is applied as a driving force. Chemical leakage is detected by appropriate instrumentation.

Sensitivity: ≥ 10-6 Pa.m3/s	
Pros:	Cons:
Usually more sensitive and quantitative than dye	Destructive
Correlates to liquid leakage	
Operator independent	

(table continued on next page)

Table 4.2 (Continued)

Reported Usage:

Metal cans (Mg++ detection), Vials (Cu++, NaCl detection)

Suppliers:

Dependent on detection instrumentation required

MICROBIAL CHALLENGE TESTS

Method: Containers are media filled and the seal is either challenged directly with microorganisms (in liquid suspension or aerosol form), or is allowed to sit in ambient storage environment. Presence of microbial growth is visually confirmed.

Sensitivity: erratic, $\geq$ 10-5 Pa.m3/s

Pros:	Cons:
FDA condoned	Insensitive
No special equipment required	Media fills are expensive in production down-time and labor costs
	Ambient storage chal-lenge requires large storage area

Reported Usage:

Widely used throughout the pharmaceutical industry

Suppliers:

None

WEIGHT CHANGE

Method: Container is filled with liquid or dessicant, sealed, stored at various stress conditions, and reweighed over time.

Sensitivity: time dependent, can be excellent.

Pros:	Cons:
Easy	Time consuming test
Directly relates to closure performance	Leak location not detected

Quantitative

Inexpensive

Reported Usage:

Vials, Aerosols, Solid dosage form packaging

Suppliers:

None

(table continued on next page)

Table 4.2 (Continued)

HELIUM MASS SPECTROMETRY

Method: Helium is placed either inside or outside of the container. Vacuum is applied to seal interface and migrating helium is detected by mass spectrometry.

Sensitivity: ≥10-12 Pa.m3/s

Pros:	Cons:
Inert gas	May confuse helium diffusion with leakage
Extremely sensitive test	
Rapid test time	
Quantitative	

Reported Usage:

Refrigeration units, Automotive parts, Pacemakers, Food & beverage containers, Drums

Suppliers:

Leybold Inficon Inc., East Syracuse, NY

Alcatel Vacuum Products, Inc., Hingham, MA

GAS DETECTION TESTS

Method: Test tracer gas is placed on one side of container seal. Inert carrier gas is passed across opposite seal side. Tracer gas is detected either by a coulometric detector (O2), or by a photo-electric sensor (CO2 or H2O). Instruments which are designed to pierce containers and test package headspace for O2 or CO2 are another type of gas detection test method.

Sensitivity: Time/instrument dependent

Pros:	Cons:
Directly relates to package performance	Slow
Does not pick up false leaks as helium detection can	Often fixture dependent

Reported Usage:

Screw-capped bottles, Food & beverage containers

Suppliers:

Modern Control, Inc. (MOCON), Minneapolis, MN

VACUUM-IONIZATION TEST

Method: High voltage, high frequency field is applied to vials sealed under vacuum. The field causes residual gas to glow. Glow intensity is function of vacuum level.

(table continued on next page)

Table 4.2 (Continued)

Sensitivity: not documented

Pros:	Cons:
On-line, nondestructive test	Some protein decom-
Rapid	position reported

Reported Usage:

Genentech, Inc. for lyophilized vials of biopharmaceuticals

Suppliers:

Electro-Technic Products, Inc., Chicago, IL

ELECTRICAL CONDUCTIVITY/CAPACITANCE TESTS

Method:

Conductivity: High frequency, high voltage is applied to seal container. Increase in conductivity correlated to presence of liquid along the seal.

Capacitance: Increase in dielectric constant across seal is detected due to presence of liquid.

Sensitivity: not determined

Pros:	Cons:
Both permit 100% inspection of product	On-line capacitance
Not operator dependent	test not yet available
Clean	
Nondestructive	

Reported Usage:

Electrical conductivity for ampules; capacitance for ophthalmic plastic bottles

Suppliers:

Electronic System Pinhole Inspector, Eisai USA, Inc., Torrance, CA (for ampules)

Bosch Pinhole Detector, Robert Bosch Corp., S. Plainfield, NJ

Modern Control, Inc. (MOCON), Minneapolis, MN
(for capacitance tester)

the few tests used for evaluation of large volume parenteral flexible containers, where the liquid-filled package is pressed, and the inspector visually checks for leaks (13). Visual inspection may be coupled with the application of vacuum to make leakage more readily observable.

Bubble Test

Bubble testing is generally performed by submerging the container in liquid, applying a differential pressure, and inspecting for bubbles. The sensitivity of this test may vary anywhere from 10-2 to 10-6 Pa.m3/s depending on the length of time given for observation, the differential pressure applied, lighting, background contrast, and the addition of a surfactant to lower the surface tension of the immersion liquid. One variation of this test consists of applying a surfactant solution directly onto the package surface rather than immersing the package in such a solution. To further improve method reliability, ultrasonic testing equipment has been advertised as a tool to detect bubbling. The major disadvantage of bubble tests for parenteral products is the need to wet the package seal, a requirement that would be unacceptable for most sterile packages. It certainly cannot be used for 100-percent leak detection in the production environment, but it may be useful as a rapid screening test for research.

In Ref. 11, 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 a surfactant immersion fluid, a dark background, high intensity lighting, a 3x magnifying lens, a differential positive pressure of 3 psi inside the vial, and a maximum test time of 15 minutes. Under these special conditions, the bubble test was able to detect leakage rates as low as about 10-6 Pa.m3/s.

Pressure/Vacuum Decay

Pressure or vacuum decay leak tests are often used for measuring leakage in many types of containers, including parenteral packages. These tests have proven especially useful in the evaluation of parenteral vials or bottles stoppered with elastomeric closures. For such packages the integrity of the seal is determined by the ability of the closure to

conform to the package opening under compressive capping and sealing forces (2).

One variation of this approach is the vacuum retention test. This method is designed to evaluate the ability of a rubber closure to maintain a vacuum. Following the evacuation of the headspace of a vial, the closure is secured in place on the vial finish area. Leakage and closure relaxation may be accelerated by autoclaving the package. The vacuum retained over time is determined using a modified vacuum gauge puncturing the rubber closure (13).

A method similar to vacuum retention is an internal pressure retention test. A positive pressure inside a vial may be established by stoppering the vial under pressure, or by subjecting the filled, stoppered package to heat using hot air, hot water immersion, or autoclaving. Containers are evaluated by observing for encrustation at the closure/vial interface or by checking for loss of pressure or leakage of vial contents (13). The closure blow-off test consists of fixturing the closed package to permit internal pressurization of the package. Pressure is applied inside the vial to determine the amount of force required to cause seal failure (14).

On-line leak testing equipment designed using the pressure/vacuum decay principle are marketed by several companies including Shibuya International, Wilco Precision Testers and others. In each case a small chamber is lowered onto the container, a pressure or vacuum is applied to the chamber, and the change in pressure or vacuum beyond an established limit triggers rejection of the container. Such test equipment is commonly used by cosmetic and food and beverage manufacturers. Shibuya International has designed equipment specifically for parenteral vial testing (Fig. 4.4). The drawback of such systems for on-line production use is that leakage near the microbial leakage specification limit of 10-6 Pa.m3/s cannot typically be detected within the short test time allowed during high-speed filling operations. However, continued research efforts may permit improvement of these systems to enable on-line detection of such low-level leaks in the future.

A laboratory test system designed for measuring leakage from parenteral vials is described in Ref. 15. The set-up consists of a

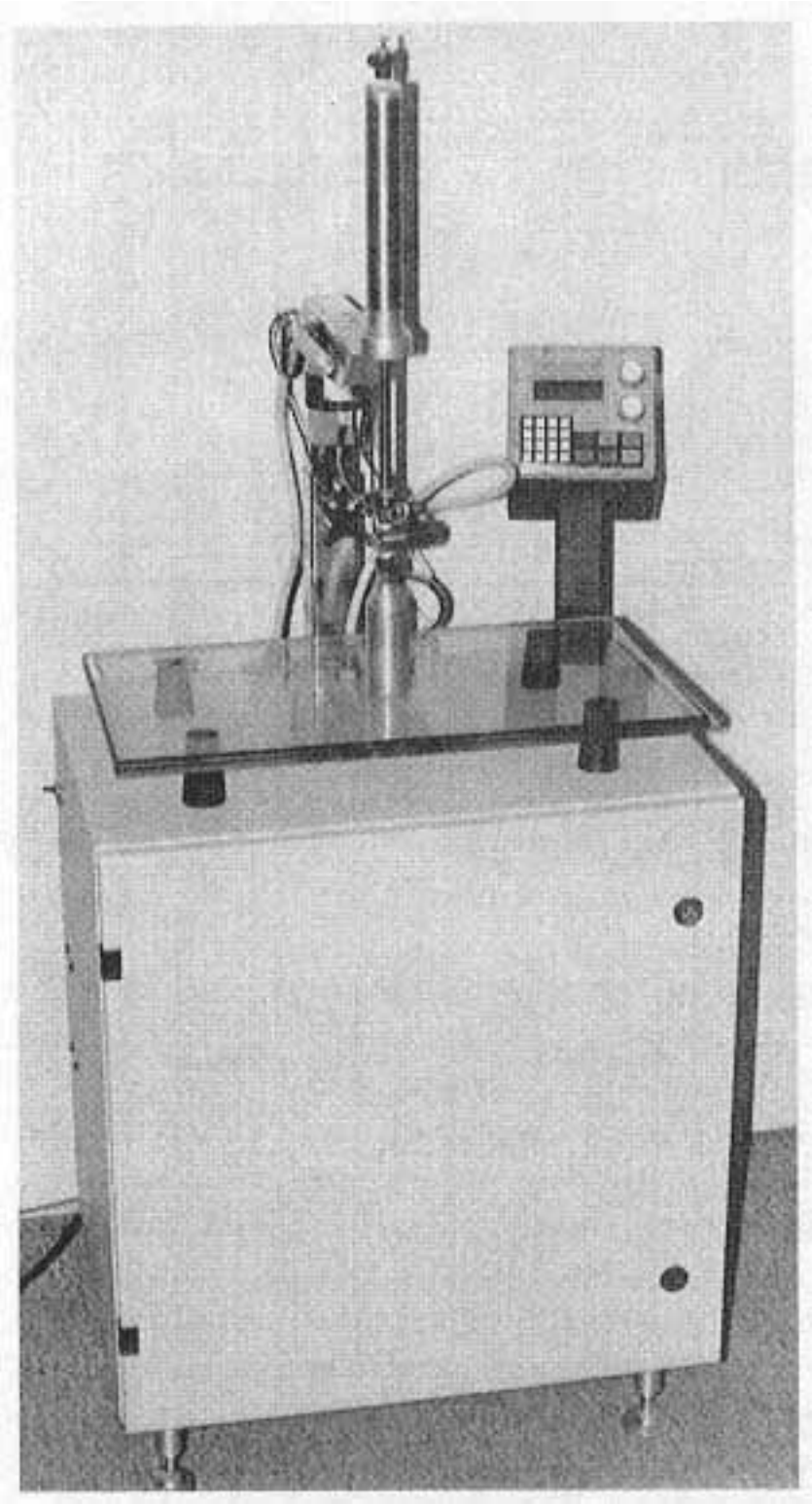


Fig. 4.4
Shibuya America integrity test unit (courtesy of Shibuya International, Inc., Medessa, California).

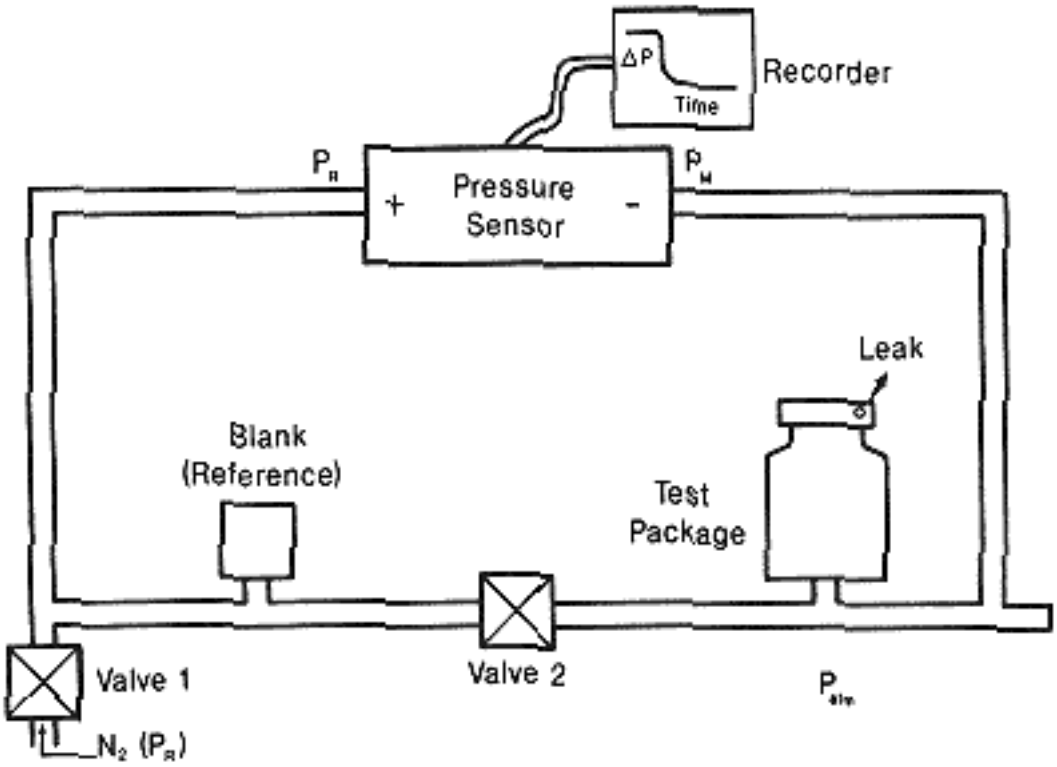


Fig. 4.5
Differential pressure laboratory test unit (courtesy of the Parenteral Drug Association, Inc., Bethesda, Maryland). Source: Ref. 15.

differential pressure transducer connected to a test manifold with a blank control side and a test half on which specially fixtured vials are attached (Fig. 4.5). With this device, stoppered vial leakage rates between 10-3 and 10-7 Pa.m3/s can be quantitatively measured as illustrated in Figures 4.6 and 4.7. The linear regressions in Figure 4.6 were generated for vials stoppered with various uncoated elastomeric closures from the residual seal force values, that is, the amount of force being exerted by the crimped closure onto the vial1 (the Y-axis), versus the percent the closure flange has been compressed as a result of capping (the X-axis). The windows in this figure indicate the minimum amount of crimp compression required to effectively seal the elastomeric closures to the vials. The vials tested had channel defects of

1For an explanation of residual seal force, see the Section Package Function and Leakage Specifications.

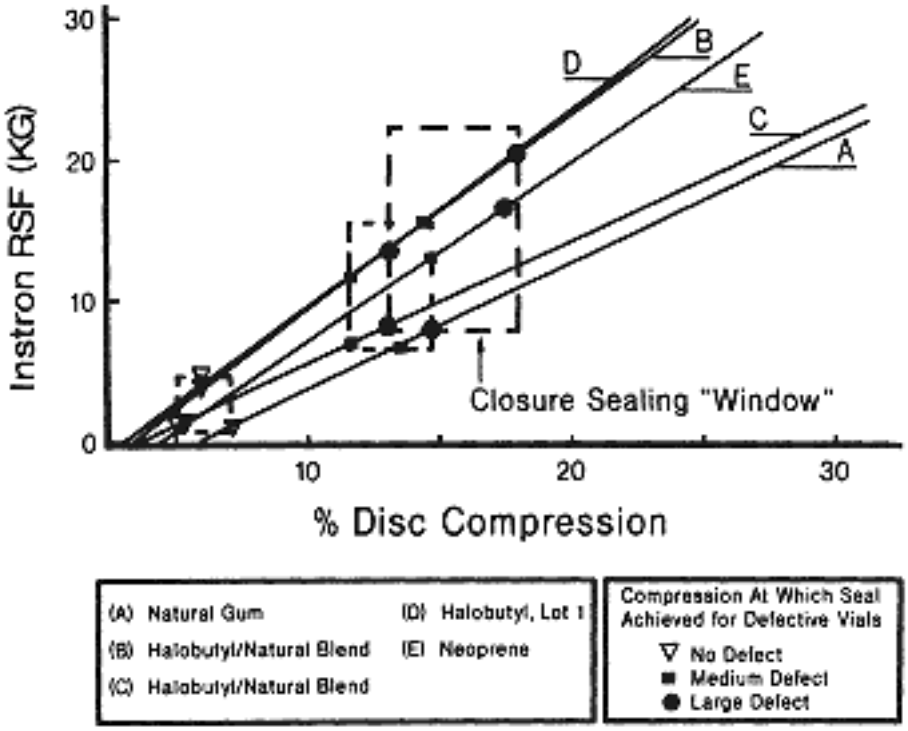


Fig. 4.6
Leakage vs. percent uncoated closure compression
on parenteral vials (courtesy of the Parenteral Drug Association, Inc.,
Bethesda, Maryland). Source: Ref. 15.

varying depth etched across the sealing surface. A vial was considered to be sealed once the leakage rate dropped below the lower limit of detection for this device (≥ 10-7 Pa.m3/s). 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.

In Figures 4.7a through c leakage rates versus percent of crimp compression are shown for nondefective vials sealed with halobutyl closures coated with various polymeric coatings (15). Again, crimp compression refers to the percent the closure flange has been compressed as a result of capping. The type and thickness of coatings such as Purcoat,2 polytetrafluoroethylene (PTFE), ethylenetetrafluoroethylene (ETFE), and modified polypropylene (N-8) on the flange

2Trademark of The West Company, Phoenixville, PA.

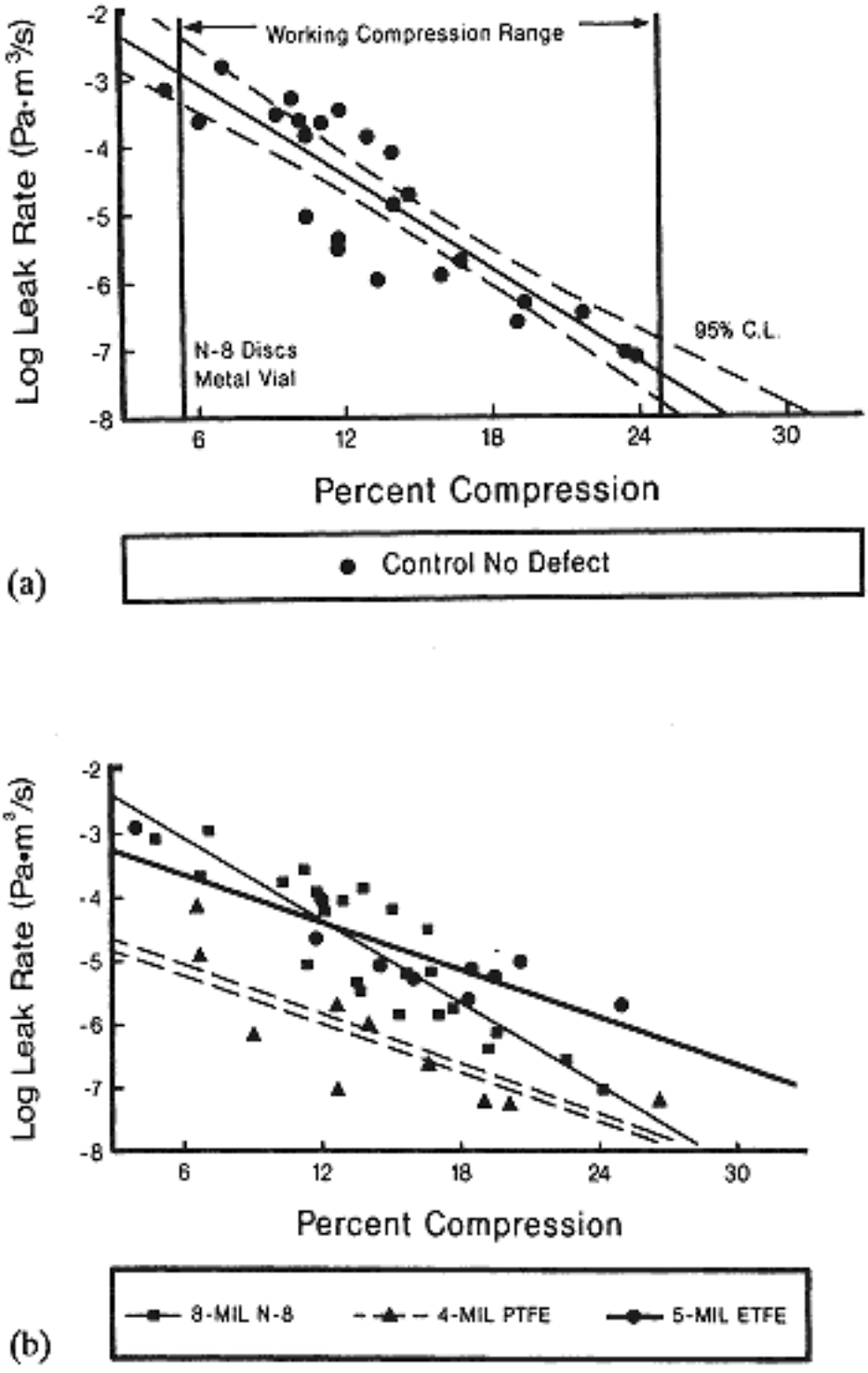
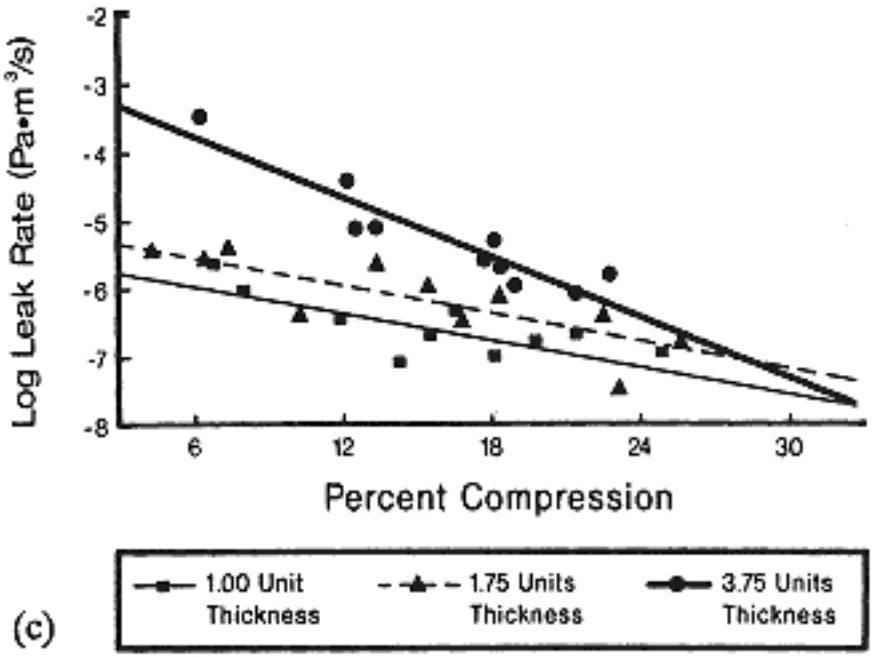


Fig. 4.7
Leakage rate vs. percent coated closure compression on parenteral vials (courtesy of the Parenteral Drug Association, Inc., Bethesda, Maryland). (a) Modified polypropylene (N-8) film-coated closure leakage rates; (b) N-8 vs. PTFE vs. ETFE film-coated closure leakage rates; (c) PurcoatTM thickness effects on leakage rates. Source: Ref. 15.



surface of the closure had a major impact on the leakage rates. Thinnly coated Purcoat closures provided the best seal of the coated closures evaluated, exhibiting a leakage rate of about 10-6 to 10-7 Pa.m3/s. By contrast, uncoated closures representing a wide range of elastomeric materials were all capable of effecting a seal demonstrating leakage rates well below the 10-7 Pa.m3/s lower limit of quantitation for this instrumentation.

Dye Tests and Chemical Tracer Tests

Movement of liquid across a seal is commonly detected by means of either a dye solution or a liquid containing a chemical tracer. Dye may be detected either visually or by means of UV spectroscopy. Visual detection of dye solutions may give highly variable results depending on operator ability.

Despite the limitations of the dye test, it remains a favorite in the pharmaceutical industry for a variety of package systems. There are several ways of conducting this test reported in the literature (13). Typically the container is immersed in a dye bath and vacuum, pressure, or a combination of both is applied. Then the container is removed and washed, and finally it is inspected for the presence of dye. Autoclaving

ampules while submerged in dye has been found to be more sensitive than applying vacuum or pressure alone, by using fluorescent dye plus vacuum, or by cooling them while submerged in dye (16).

Preferred attributes of a dye are that it be 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 cleaning solution, and easily disposed of to meet environmental safety requirements. Blue dyes are most commonly used, while green to yellow-green are more easily detected by the human eye. Blue, violet, and red dyes are the colors least easily seen. The dye test can be optimized by use of a surfactant or a low viscosity fluid in the dye solution to increase capillary migration. A typical dye solution formulation may also contain a preservative to prevent microbial growth (6,13).

Solutions containing radionuclides such as technetium 99m or chromium 51, either with or without a dye, have also been used for detection of leakage in glass ampules (17). In these studies by Butler, ampules were challenged using test solutions containing both dye and radionuclide and were examined visually for the presence of dye and by scintillation counting for the presence of radioactivity. It was found that Technetium 99m was detected in 10.2% of the ampules, which failed to demonstrate discernable color. However, many fear that a radionuclide test may be time consuming and may require special safety precautions for handling, storage, and disposal of radioisotopes.

Chemical tracer solutions may be used in place of dyes. Chemical tracer tests are somewhat more sensitive and quantitative since detection techniques such as atomic absorption or HPLC can be used. Morton et al. tested simulated parenteral vials for liquid leakage of a copper sulfate tracer solution by an atomic absorption detection assay (11). A saturated aqueous solution of copper sulfate with surfactant was filled into the vial. The vial finish area was lowered into 10 mL of water while the vial was pressurized internally at 3 psi for 15 minutes. The water was tested for the presence of copper by atomic absorption. This test was calculated to be capable of detecting as little as 0.1 microliter of copper solution. The practical sensitivity of this test was compared to a bubble

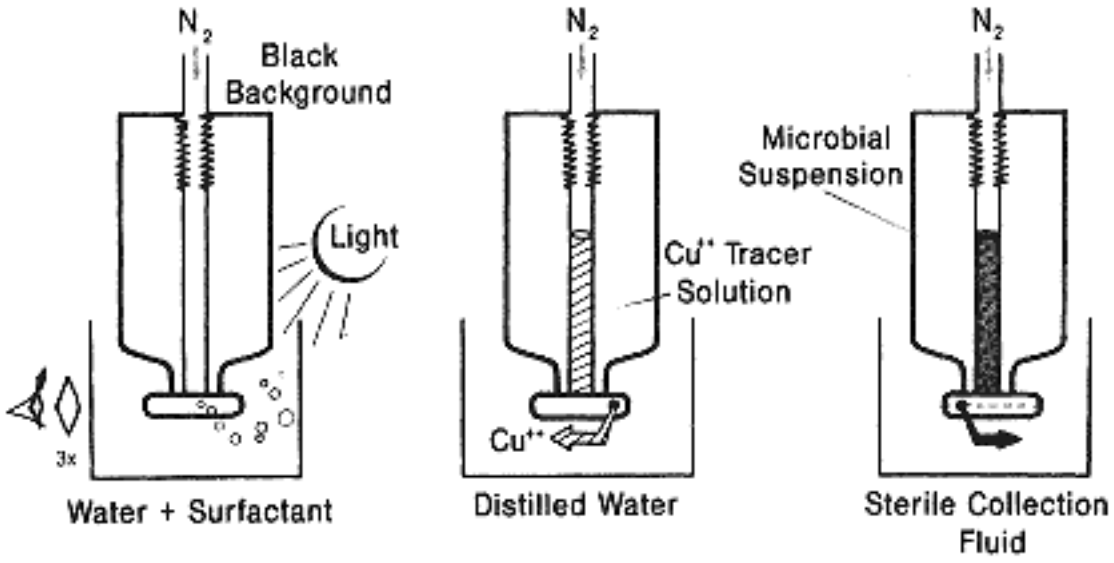


Fig. 4.8
Laboratory test units for bubble, liquid chemical tracer, and microbial egress leak test comparisons (courtesy of the Parenteral Drug Association, Inc., Bethesda, Maryland). Source: Ref. 11.

test, a microbial challenge test, and gaseous leakage quantitatively measured by the laboratory pressure decay device described on pages 265 and 266 (Fig. 4.8). It was found that leakage of the chemical tracer solution was detected in all containers known to exhibit gaseous leakage rates greater than or equal to 10-5 Pa.m3/s. This is consistent with literature references that state that aqueous leakage will not occur when dry air, at the same pressure, leaks at a rate as great as 10-5 Pa.m3/s (10).

A similar study was performed using metal cans in which microbial ingress was measured and correlated to water ingress and helium gas leakage rate (18). 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 logarithmically related to the log of the helium gas leakage rate measured through the cans.

Microbial Challenge Tests

The only package integrity test officially recognized by the FDA for parenteral containers is the microbial challenge test. In this test

containers filled with either product or a culture media are exposed to microorganisms and subsequently evaluated for sterility. This approach to validating package integrity has been condoned by the FDA and is extensively used by the pharmaceutical industry to directly correlate package defects to microbial contamination risk.

Ironically, microbial challenge and sterility tests yield erratic results and may not be capable of detecting even liquid leakage, much less lower level gaseous leakage which is critical for packaging that requires an inert or dry atmosphere. Microbial leakage tests are tedious, difficult to perform, and necessitate a large database to minimize error due to false-positive and -negative results. A more quantitative leak test procedure using either liquid or gas as a tracer is more accurate and predictive of critical leakage of microbial dimensions. Because of the ineffectiveness of microbial challenge and sterility tests, such techniques have more value when coupled with other quantitative physical leakage tests and/or with package assembly and performance assessments.

There are three basic variations to microbial challenge tests (2,14,19). *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, an added stress of vacuum and/or pressure cycling may be used (*dynamic vs. static testing*). The third type of test is called the *static ambient test*. It involves the storage of packaged media or product under typical warehouse conditionsthe stored containers are checked for evidence of microbial ingress over time.

Within each type of test there are many variations in use, some of which are described more fully below. Each test option 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.

Immersion Tests

Very simply, the immersion test involves terminally sterilizing broth- or product-filled packages followed by immersion in an aqueous suspension of microorganisms for several minutes to an hour. The entire

package or only the seal area may be immersed (Fig. 4.9) (14). The packages are then incubated and nonsterile containers are visually identified.

In designing an immersion test it should be remembered that terminal sterilization will change the nature of some types of package seals by, for example, relaxation of elastomers or distortion of thermoplastic materials. Therefore the sterilization cycle should duplicate that actually used for the product. If this is not possible, as in the case of aseptically filled product, it would be desirable to demonstrate that the seal of the terminally sterilized test package does not differ appreciably from that of the aseptically filled product. When aseptically filled packages are tested, only nonsterile containers shown to be contaminated with the

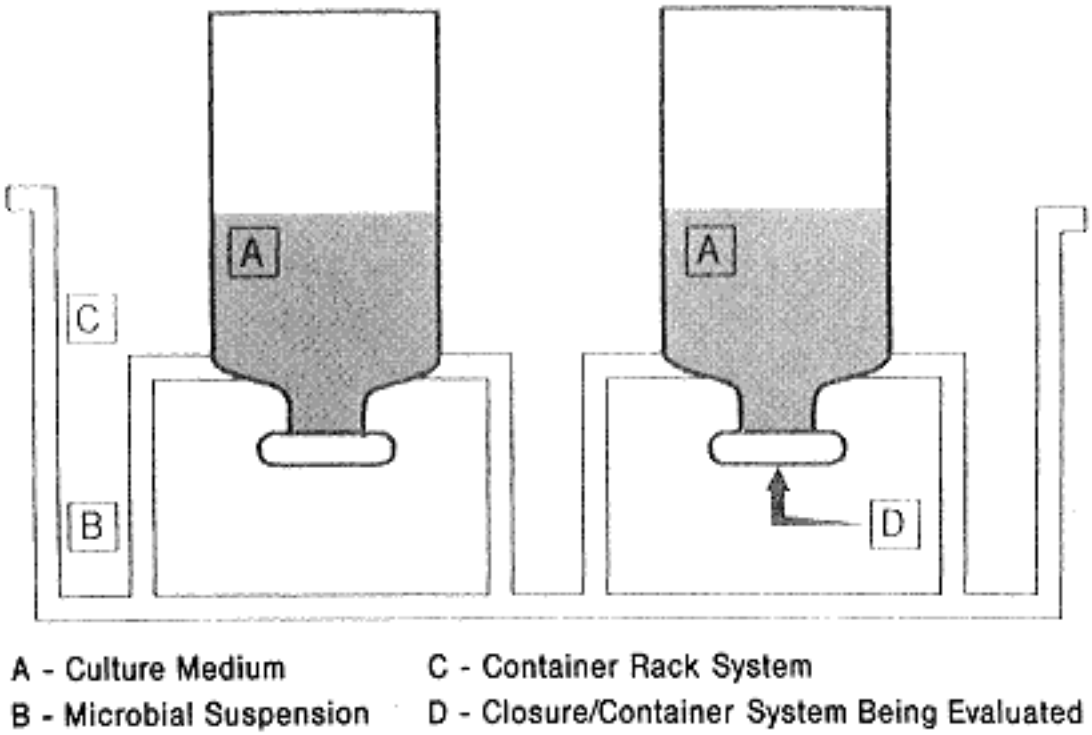


Fig. 4.9
Container/closure system microbiological immersion test (courtesy of Interpharm Press, Buffalo, Grove, Illinois). Source: Ref. 14.

challenge microorganisms are generally considered a true positive leaker.

An additional test variable to consider is the selection of the challenge microorganism. Examples of those reportedly used for such tests include *Pseudomonas aeruginosa* (11,14), *Pseudomonas diminuta* (13), *Enterobacter coli* (20), and *Serratia marcescens* (21). Size, motility, and viability in the product or culture media are all factors in microorganism selection. Culture media likewise vary depending on the nature of the microorganism used. Vacuum and/or pressure cycles may be included to further stress the package to mimic product processing challenges such as autoclaving or shipping and storage conditions. The package is then incubated at temperatures most appropriate to promote growth of the given 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 (14). Nonsterility is evidenced by cloudiness in the package, or in the case of product-filled packages, verification of nonsterility may require aseptic filtration and filter plating for microorganism identification. Any nonsterile package contaminant is generally identified to verify the challenge microorganism as the source of contamination.

The sensitivity of a microbial immersion challenge test was compared to a bubble test, to liquid leakage via a chemical tracer solution, and to a quantitative pressure decay leakage test, using simulated parenteral vials stoppered with closures film-coated with modified polypropylene (11). Descriptions of the bubble, chemical tracer, and pressure decay tests included in this reference can be found in earlier sections of this chapter. To perform the microbial challenge test, the test vial was filled with a suspension of *Pseudomonas aeruginosa* at a minimum concentration of 3 × 108 colony-forming units/mL. The vial was then inverted and immersed in 10 mL of sterile saline and pressurized internally at 3 psi for 15 minutes (Fig. 4.8). The saline was evaluated for the presence of the challenge microorganism by a filter plate count method. Figure 4.10a illustrates the incidence of microbial leakage for vials sealed at various closure sealing compression levels superimposed on the gaseous leakage rates measured by differential

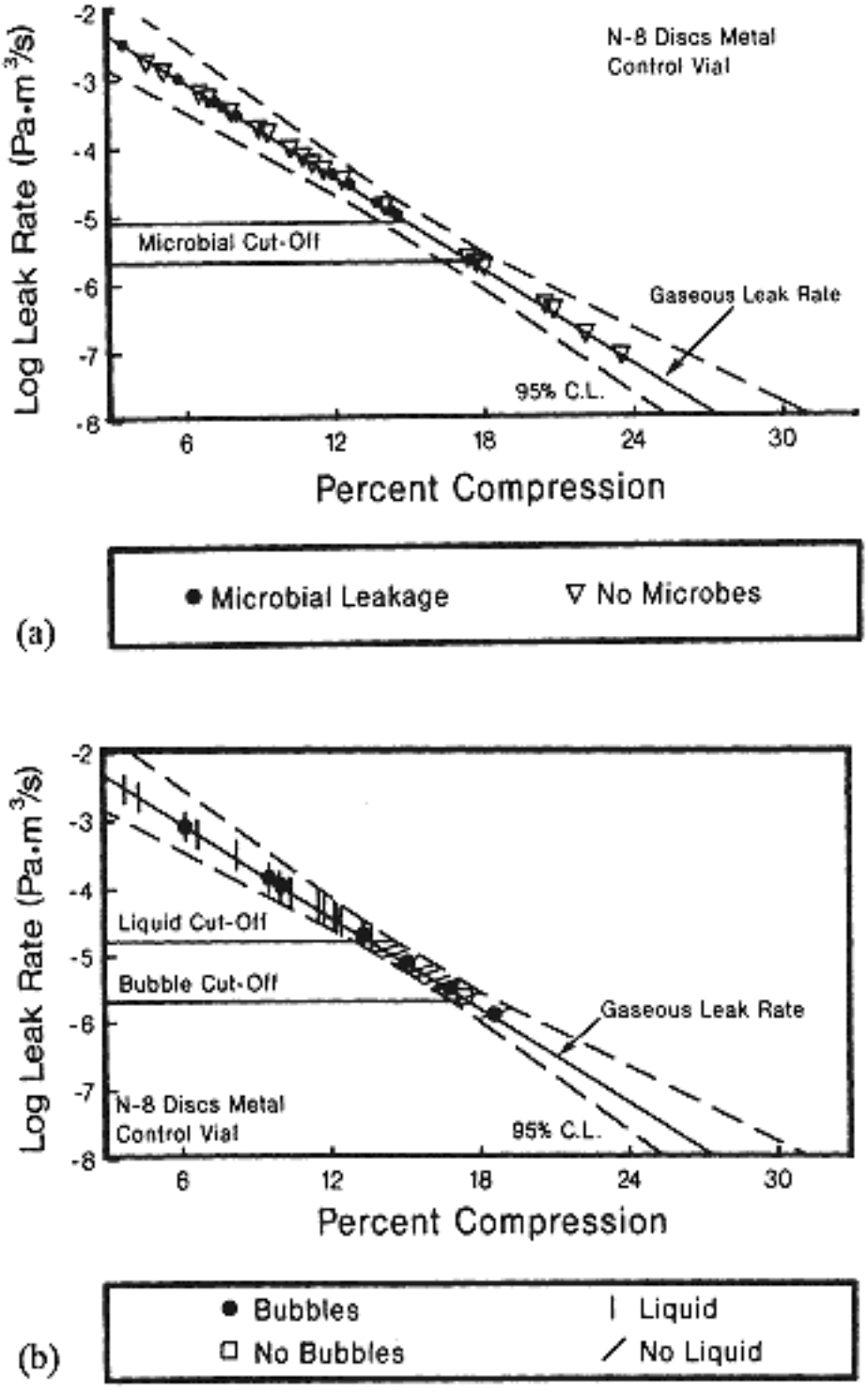


Fig. 4.10
Parenteral vial leakage test sensitivity results (courtesy of the Parenteral Drug Association, Inc., Bethesda, Maryland). (a) Microbial leakage vs. gas leakage; (b) liquid chemical tracer and bubble-test leakage vs. gas leakage. Source: Ref. 11.

pressure decay. Vials sealed at higher compressions appear to effect a microbial sound seal, corresponding to gaseous leakage of about 10-5 Pa.m3/s to 10-6 Pa.m3/s. This leakage rate is near the limit of detection for the liquid chemical tracer test (Fig. 4.10b). However, above this microbial cut-off point, movement of microorganisms occurred sporatically across the seal, with microbial colony counts for the leakers having no relationship to the gaseous leakage rate. These erratic results are not surprising given the large size of the microorganisms compared to molecules of liquid or gas and the tortuous path the organisms must trevass.

Aerosol Tests

Packages evaluated by the aerosol test are placed inside a confined vessel or chamber where they are challenged with a nebulized cloud of microorganisms (Fig. 4.11) (14). Pressure dynamics may be incorporated to simulate shipping or processing conditions anticipated by the product or simply to further stress the package system to optimize microbial ingress. This is a less stringent test than the immersion test, as it uses a lower concentration of microorganisms (generally about 103 microbes per cubic foot vs. 106 to 108 colony-forming unit per mL for immersion tests), and there is no liquid present to help carry the organisms through the leakage path. For these reasons it will also be less effective at detecting a leakage pathway. However, this may be a more realistic challenge test for those packages which contain no fluid and rarely, if ever, are wetted in actual manufacture or use.

Considerations in 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 must also 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 Reference 14.

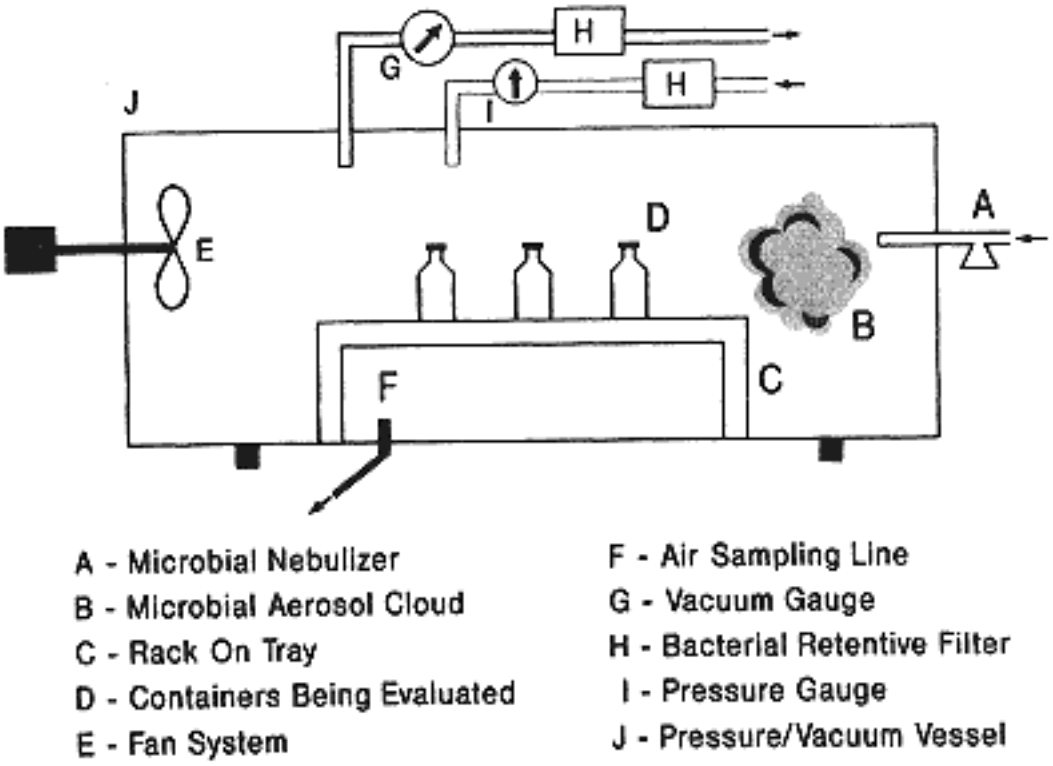


Fig. 4.11
Microbial aerosol test system to evaluate container closure systems (courtesy of Interpharm Press, Buffalo Grove, Illinois). Source: Ref. 14.

Static Ambient Tests.

Static ambient tests involve placing the product of media-filled packages on storage and evaluating them over time for sterility. This type of test is frequently performed using packaged culture media taken from the filling validation run for an aseptically filled product. Product stored and tested at expiry for sterility is also a type of static ambient test. Because there is no concentrated microbial challenge to the product, this type of test is the least effective at detecting leakage. Actual or simulated shipping tests on the filled package may be included as an additional challenge of the package system.

Weight Change

In the weight change test methods, the container is filled with either liquid or desiccant, sealed, and stored at various possible conditions of

relative humidity and temperature. The weight gain or loss is measured over time. This test is recognized in the USP for evaluation of solid pharmaceutical dosage form packaging. It is quite accurate, simple to perform, and is extremely useful for direct correlation to packaged product performance. The major drawbacks for this technique include the extended time required to perform the test, and the fact that it does not readily permit leakage location.

The time required for weight-loss detection can be expedited by storage at elevated temperatures or by filling containers with materials that more rapidly lose weight. For instance, dry ice has been used in weight-loss tests of intravenous screwcapped bottles (22), and isopropanol has been filled into parenteral vials stoppered with closures at various compression levels (2). 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, or desorption from, the packaging materials themselves.

Helium and Other Gas Detection Tests

Leak test methods based on detection of gaseous molecules are generally the most sensitive of all leak test methods, with helium mass spectrometry being the most sensitive gas detection technique of all. In fact, helium molecules are so effective at traversing through pores that helium diffusion through materials may actually be confused with leakage. Other gases frequently used for leakage detection of pharmaceutical, food, and cosmetic packages include oxygen, carbon dioxide, and water vapor. The sensitivity of all gas detection methods generally depends on the test instrument design and capability and the allowed test time.

Helium detection by mass spectrometry can be performed by putting helium either inside or outside the package. Any helium leaking through the system is pulled by vacuum into the unit and is detected by mass spectrometry. Vacuum sampling can be done by use of a probe device that is manually swept across the package surface; or the entire

package can be placed in a high vacuum test chamber. This technique is extremely rapid and can accurately detect leaks as small as 10-12 Pa.m3/s.

A helium-spark method has also been used to detect leakage in ampules (13). By this procedure the container is sealed under a helium headspace followed by a vacuum treatment for several minutes. The ampule is then brought near a high frequency spark coil that will cause a blue discharge if helium is present. Although this technique claims to detect pinholes as small as 7 microns, it is suggested that larger leaks would be overlooked due to the rapid removal of the tracer gas during the vacuum cycle.

Modern Controls, Inc. (MOCON) provides a variety of instrumentation for detection of oxygen, water vapor, and carbon dioxide (Fig. 4.12). Regardless of the detection gas, the test units are similarly designed. A carrier such as nitrogen is flushed through or around an appropriately fixtured package. Any oxygen, carbon dioxide, or water vapor that diffuses through the package seal is detected by an appropriate sensor. Oxygen is detected by a coulometric detector, and water vapor and carbon dioxide are detected by a photoelectric sensor. MOCON as well as other instrument companies market equipment capable of directly testing the container headspace for oxygen or carbon dioxide using a sample probe designed to pierce the container wall or closure.

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 be evaluated using carbon dioxide test equipment. Helium detection is usually reserved for very high sensitivity testing needs.

Vacuum-Ionization Test

The vacuum-ionization test, or spark-coil test, is useful for vials or bottles sealed under vacuum. A high voltage, high frequency field is applied to the vials, which causes residual gas to glow. The glow intensity is a function of headspace vacuum level, with a blue glow indicative of vacuum and no glow to a very faint or purple glow indicative of no vacuum (Fig. 4.13). It has reportedly been used for

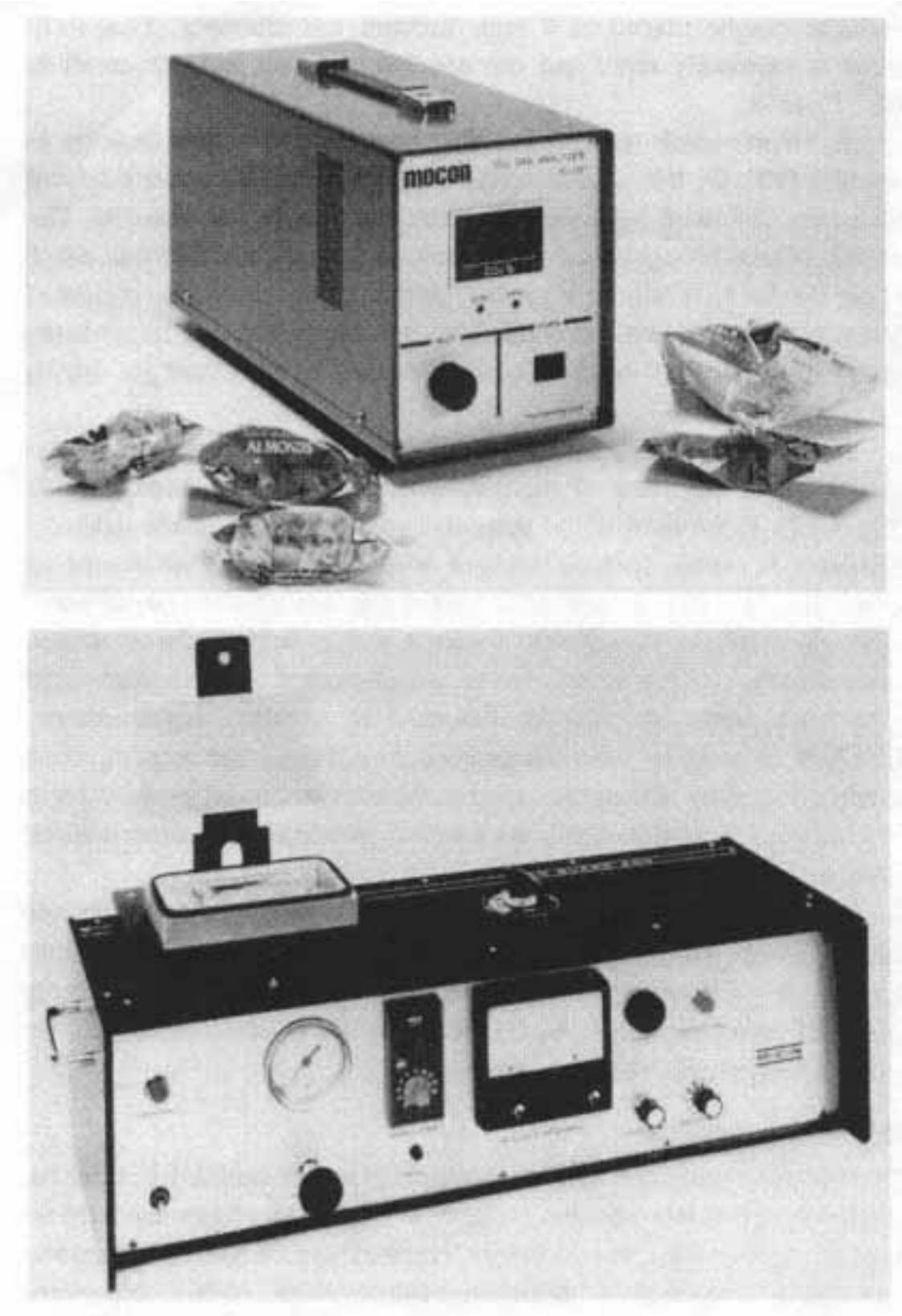


Fig. 4.12
Leak test instrumentsCO2 gas analyzer and the PAC Guard 400 package tester (courtesy of MOCON, Modern Controls, Inc., Minneapolis, Minnesota).

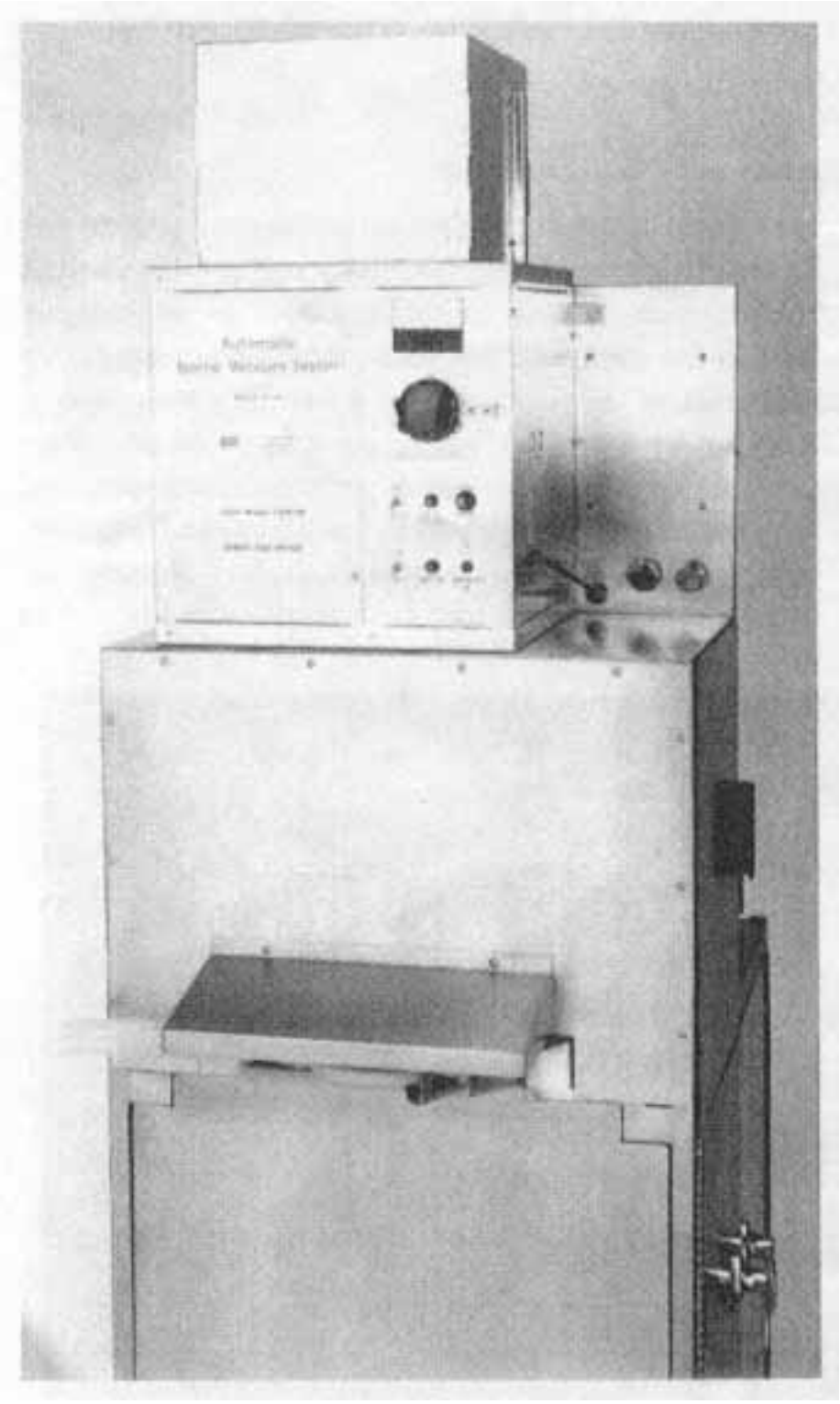


Fig. 4.13
Electro-Technic Products, Inc. automated vacuum bottle testing system (courtesy of Electro-Technic Products, Inc., Chicago, Illinois).

on-line testing of lyophilized products, however the potential for protein decomposition requires that the method be validated for product compatibility (23).

Electrical Conductivity and Capacitance Tests

Glass ampules are often tested using on-line electrical conductivity test equipment (2,13). By this method a high frequency voltage is applied to the container. Any presence of liquid indicating a break in the glass, or possibly a thin area in the glass wall will allow increased conductivity and subsequent rejection of the package (Fig. 4.14). This technique is rapid (up to 18,000 units per hour), clean, capable of on-line non-human inspection, able to detect very small pinholes (minimum size claimed is 0.5 micrometer), and applicable to amber-colored glass. Its primary disadvantage is the difficulty in quantitatively validating the

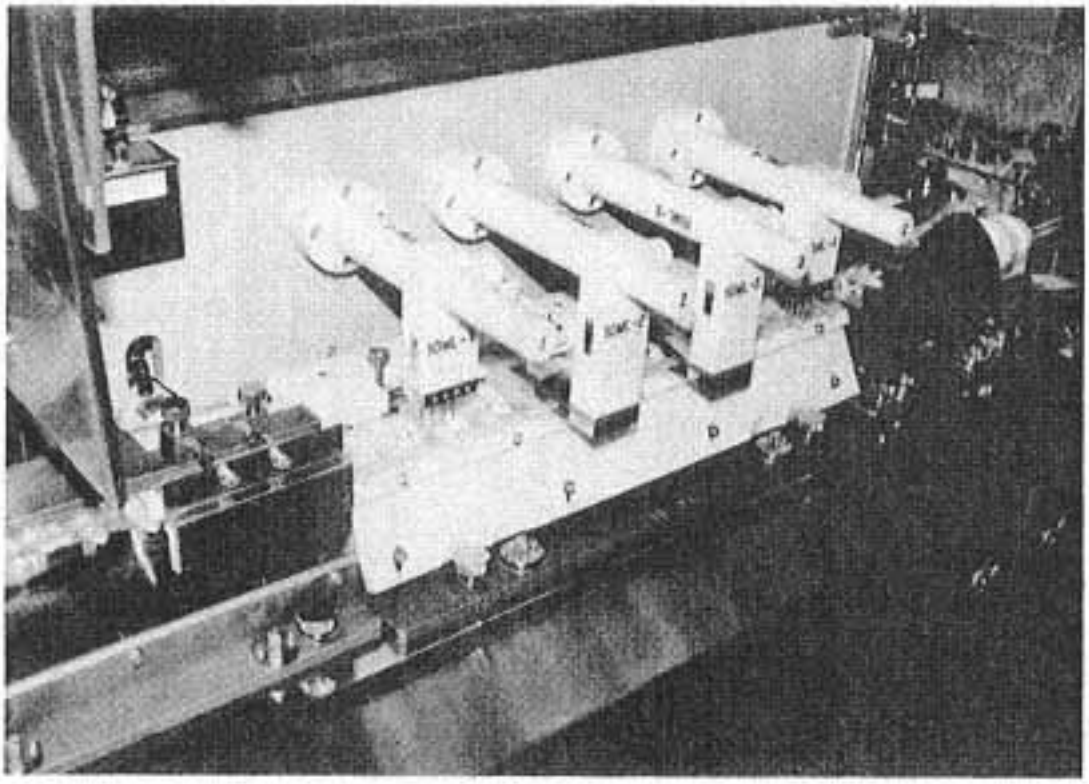


Fig. 4.14
Electronic system pinhole inspector (courtesy of Eisai USA, Inc., Torrance, California).

unit's accuracy. A creative way of correlating conductivity to detection sensitivity and reproducibility of the Eisai HDA II was performed by Sandoz AG using test ampules made of Teflon with defined electrical resistors inside them (24). More often, pinhole detectors are simply adjusted to cull out leakers detected by some other less sensitive technique such as the dye immersion test. Eisai USA also markets leak detectors for glass vials and prefilled syringes. No literature references on validation of this leak detection method for these packages are currently available.

A similar concept was utilized by Ambrosio and Jagnandan (2) in conjunction with MOCON, Inc. to develop an electrical capacitance test instrument for ophthalmic plastic bottles. The filled capped bottles are placed in an inverted position to allow liquid to flow into any crack in the plastic dropper tip. A small electrical charge is passed across the cap area of the container and the presence of liquid in the defective tip results in a greater dielectric constant reading than that of a nondefective plastic tip. This leak test method would appear to be widely applicable to a variety of plastic containers of liquid products.

The Changing Pharmaceutical Industry

The pharmaceutical industry has grown comfortably familiar with traditional parenteral dosage form package systems. Glass ampules, stoppered glass vials or bottles, plastic ophthalmic solution dropper tip bottles, and prefilled syringes have been successfully manufactured for decades. The integrity of these systems has been validated by years of experience dedicated to fine tuning both the package and its assembly. But the introduction of novel package designs along with a changing regulatory environment may force the industry to rethink its approach to package development.

A plethora of package designs and materials of construction are now available to the pharmaceutical manufacturer. Package designs have become more complex to make for easier preparation and administration of parenterals, such as the ADD-Vantage vial/flexible container admixture system marketed by Abbott Laboratories (Fig. 4.15). Examples of other novel packaging/delivery systems include unit-dose

Fig. 4.15
Abbott ADD-Vantage
package system (courtesy
of Abbott Labs, North Chicago,
Illinois).

insulin syringe cartridges and portable intravenous infusion systems. Lyophilized products or powders for reconstitution may utilize wet/dry combination packaging, for example the Lyo-Ject II syringe by PharmaTurm, Inc., or the HYPAK Liqui/Dry dual chamber prefilled syringe system by Becton Dickinson Pharmaceutical Systems (Fig. 4.16), or the Redi-Vial formerly marketed by Eli Lilly (Fig. 4.17). Similarly, wet/wet combination systems may be used to separate liquids, such as the Heparin Dextrose double bag product by Baxter Healthcare (Fig. 4.18). Packaging materials have also continued to evolve to include

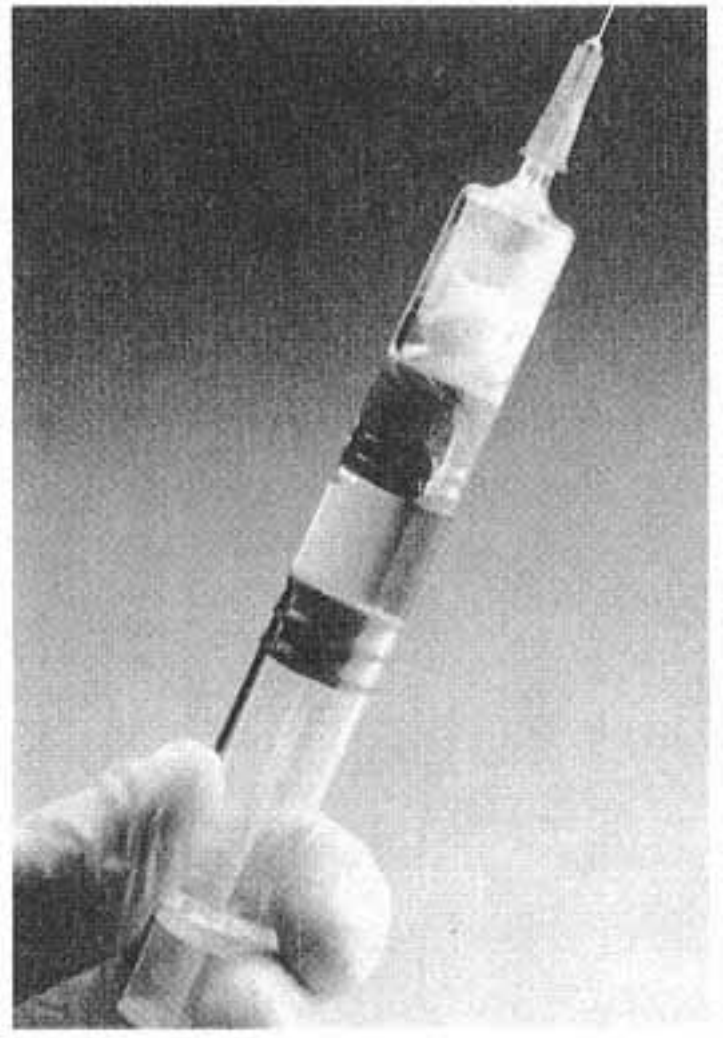


Fig. 4.16
HYPAK liqui/dry dual chamber pre-filled syringe system (courtesy of Becton Dickinson Pharmaceutical Systems, Fair Lawn, New Jersey).

elastomeric closures coated with more inert, lower particulate shedding polymeric materials, thermoplastic closures, and newer plastics for vials, bottles, and flexible containers, for example.

As packaging designs and materials change, so will the typical techniques for sterilizing packaged product and package components. Gamma irradiation, E-beam sterilization, and hydrogen peroxide gas may become more viable options. Advances in steam sterilization equipment have made possible air-over-steam cycles and high or ultra-high

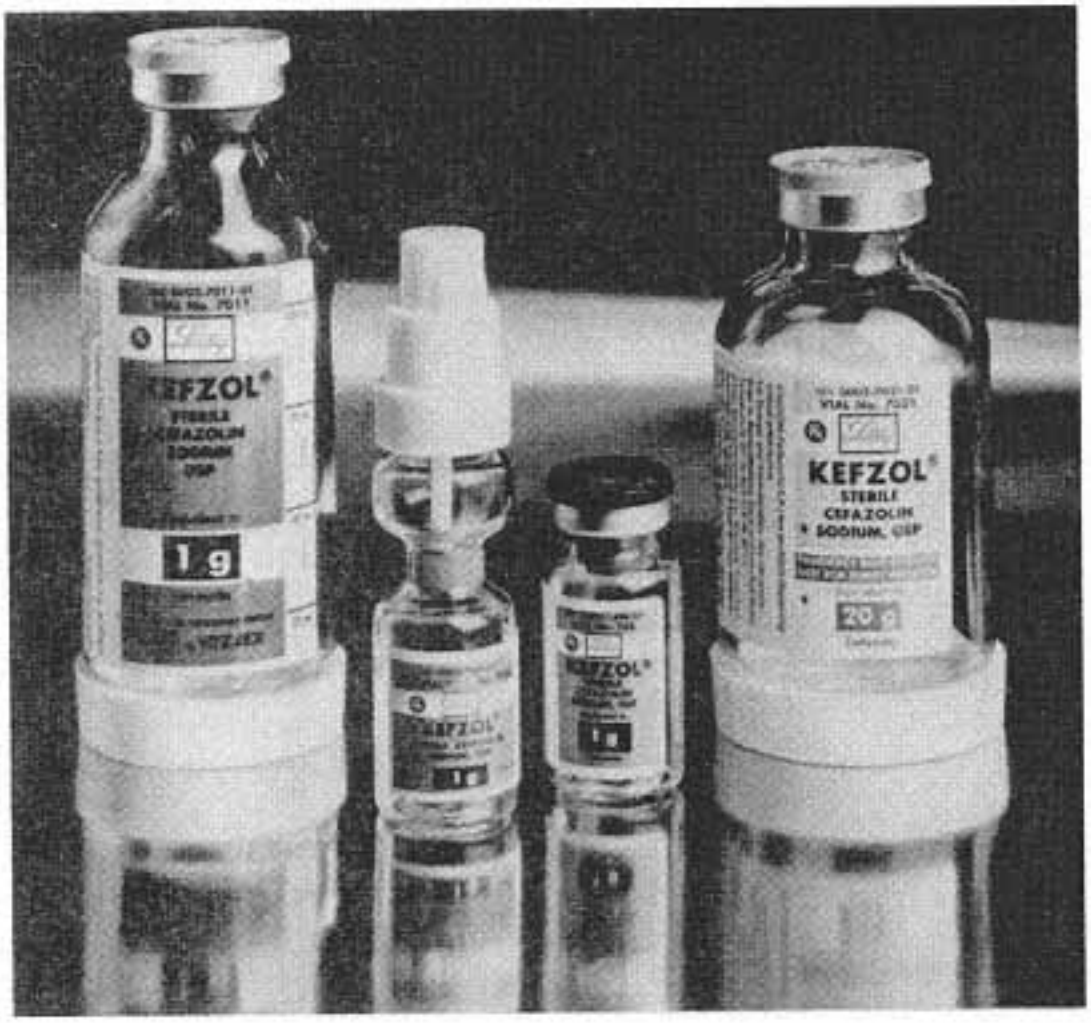


Fig. 4.17
Redi-Vial dual chamber package included with the Kefzol
product line (courtesy of Eli Lilly and Co., Indianapolis, Indiana).

temperature cycles, which permit more controlled sterilization of packages prone to blow-out and more rapid sterilization of marginally thermal stable chemical compounds.

The FDA has now provided an added incentive for more intensive research. Recently the FDA proposed that all new parenteral products be terminally sterilized or sufficient data must be provided to demonstrate that terminal sterilization is not a workable option. Several other countries have already mandated terminal sterilization whenever possible

Fig. 4.18
Heparin dextrose double bag
(courtesy of Baxter Healthcare Corp.,
Round Lake, Illinois).

(25). To sufficiently evaluate the effects of terminal sterilization on parenteral product packages, it is important that the integrity of the package be thoroughly checked under the worst-case conditions of package design, assembly, and sterilization.

Package Development Approach

In light of all this change, it is certainly advisable to follow a carefully outlined approach to package development to ensure a rational selection

of packaging components and processing and assembly variables that will ultimately minimize package integrity failures. Package integrity validation should include testing at specification limits for each critical variable. Whenever possible, it is also worthwhile to allow the component or process to roam outside the specifications to permit the determination of the edge of failure. Once the relationships between leakage rate and package materials and dimensions, processing and assembly parameters, and distribution are defined, further leakage tests should only be necessary to demonstrate control of the processes and the package.

Package Function and Leakage Specifications.

The first consideration in designing a parenteral product package is to define the intended functions of the package. All parenteral packaging must maintain the sterility of the contents. But 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 must also function appropriately to deliver product to the patient. For example, a prefilled syringe must breakaway and extrude with reasonable force and without leakage of the contents, the CRIS or ADD-Vantage systems must mate without contamination or loss of product, and a stoppered multi-dose vial must reseal upon puncturing and not core.

Once these functions have been identified, leak rate specifications can be established. For example, a container that must simply maintain sterility and prevent the loss of the liquid contents needs to meet a leak rate specification of 10-6 Pa.m3/s, as discussed earlier in the Section Establishing Leak Rate Specifications. 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 lyophilized

or powder-filled vial may need to meet rather strict gaseous leakage specifications during its shelf-life. But once mated with a CRIS flexible container, it is only important that no liquid leakage occurs 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 include physical tests designed to provide rapid, clear, and reliable results for screening packages throughout the development process.

Packaging Selection

The next phase involves the selection of appropriate packaging materials and packaging component designs. Materials are first screened based on physical and chemical compatibility with the product as well as their ability to withstand sterilization. Performance criteria should also include the ability of the material to ensure adequate package integrity. The dimensions of the components must be specified according 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 Filling and Assembly

During production, the product is filled into the package and the package is closed. This assembly process may include, for example, the torquing of a cap onto an ophthalmic bottle, the crimping of an aluminum seal onto a stoppered vial, or the flame sealing of 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

(Fig. 4.19). Screw-capped containers need to be tested for cap removal torque and leakage over time as a function of initial capping torque force.

Further verification may include components made at the dimensional specification limits, and multiple component lots. 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 post-assembly processes such as terminal sterilization on package integrity should be considered as well.

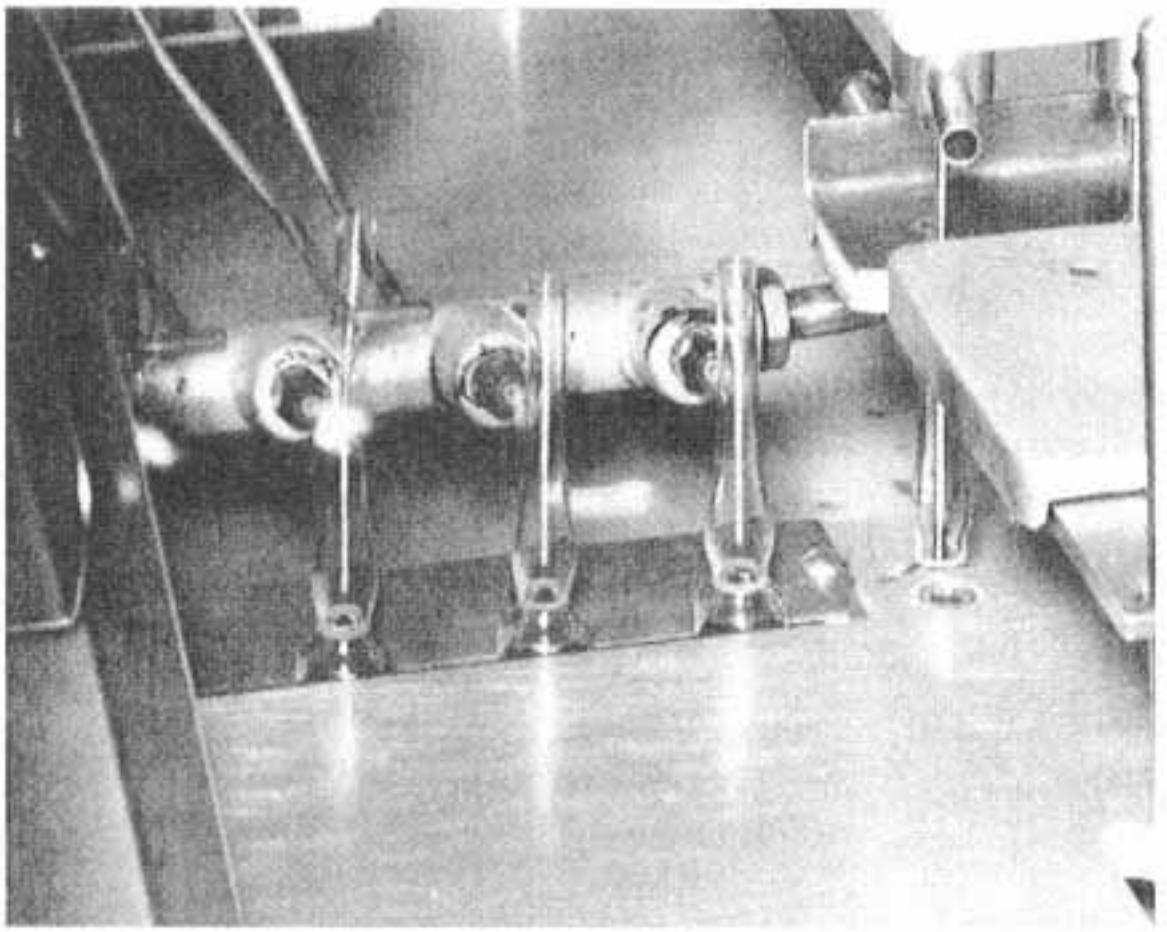


Fig. 4.19
Ampule pull-sealing operation using a filling and sealing machine
(courtesy of Cozzoli Machine Co., Plainfield, New Jersey).

The parenteral vial serves as a useful illustration of how package assembly and processing variables correlate to package integrity. Effective sealing of vials is obviously 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 too short seal skirt 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 a crimping force which act to compress the stopper onto the top of the vial. Some consistency in capping is possible with on-line capping equipment such as the West Seal Force Monitor (Fig. 4.20). Instrumentation of vial sealing equipment has also been attempted using commercially available computer software packages (26).

After capping package integrity is ensured by the compressive force exerted by the closure onto the vial finish. This force is defined as the *residual seal force* (RSF). Due to the viscoelastic nature of closures, the RSF will decrease as a function of time, processing procedures, and elastomer composition. RSF values can be determined using a West Tester, or more quantitative measurements are possible with a constant rate of strain tester (27). With the West Tester, the capped vial is compressed and downward displacement of the aluminum seal is visually observed once the compressive force exceeds the RSF (Fig. 4.21, 4.22). With the constant rate of strain tester, the capped vial is slowly compressed, and a stress-deformation response curve is generated from which the RSF value can be determined (Fig. 4.23). Ludwig et al. have cited that more reproducible curves are possible using a modified constant rate of strain test fixture (28). With the Ludwig method, the metal cap anvil has a rounded top designed to prevent uneven compression of slightly imperfect vials during testing, and it fits more snugly onto the vial cap to improve centering of the cap anvil onto the vial.

Figure 4.6 illustrates the range of RSF values determined for a variety of elastomeric formulations, while Figure 4.24a shows the range

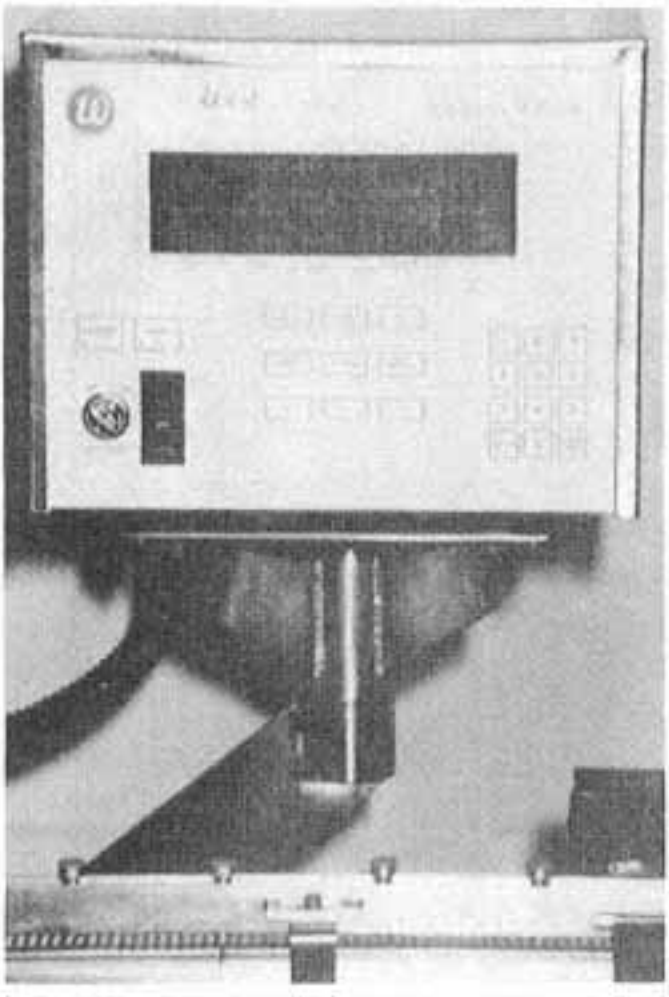


Fig. 4.20
The West Seal Force Monitor,
model WG-008 (courtesy of The West
Co., Phoenixville, Pennsylvania).

of RSF values seen among three lots of the same formulation. Figures 4.24b and c demonstrate how factors such as time and terminal steam sterilization can change RSF values (29). Because residual seal force is a direct indication of how tightly a parenteral vial is sealed, it is possible to correlate this physical test to package leakage rate (Fig. 4.6) (11). Thus this test can serve as a rapid, nondestructive way to monitor the package assembly operation and indirectly ensure package integrity over shelf-life.

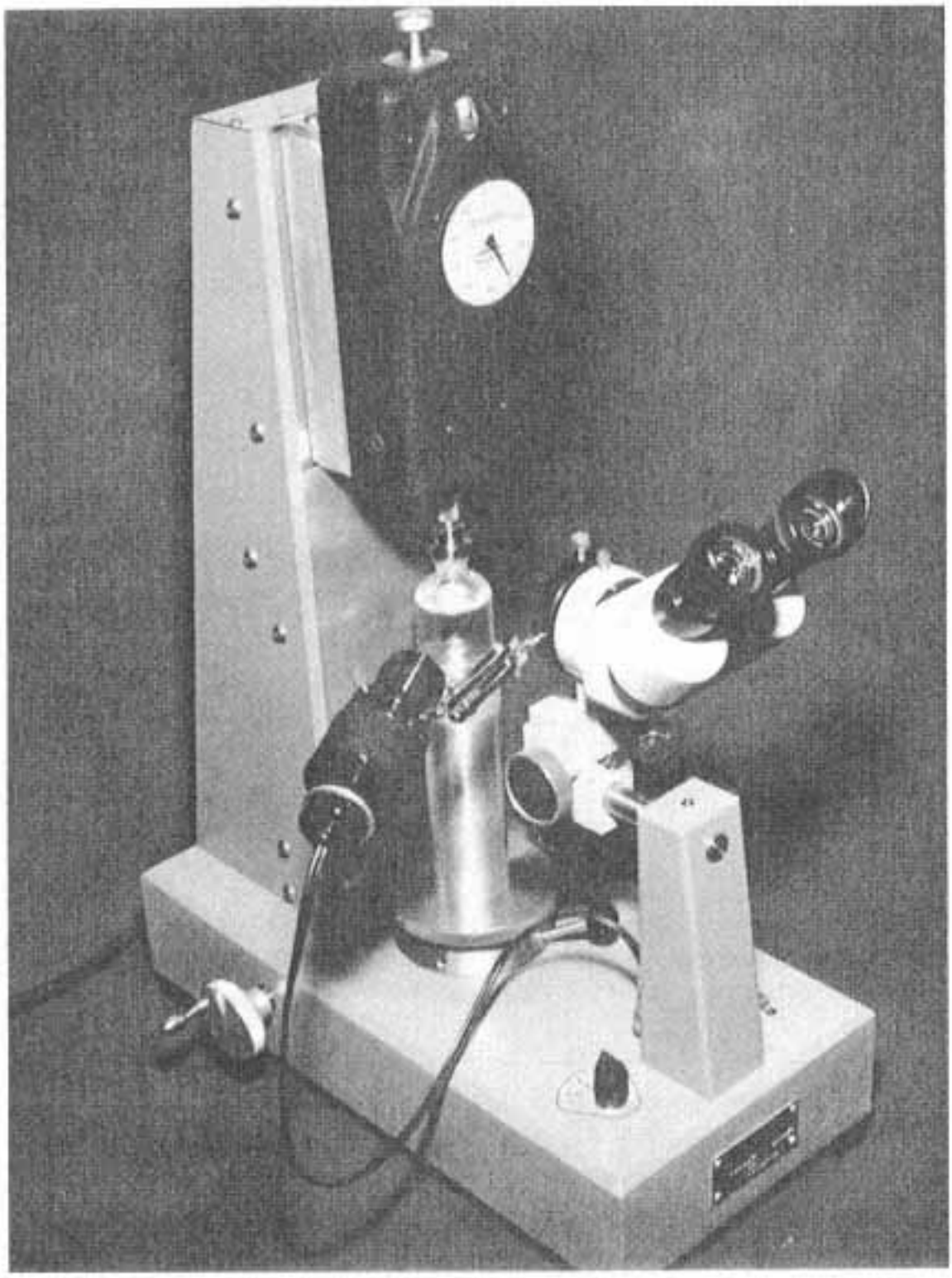


Fig. 4.21
The West Seal Force Tester, model WG-005 (courtesy of The West Co., Phoenixville, Pennsylvania).

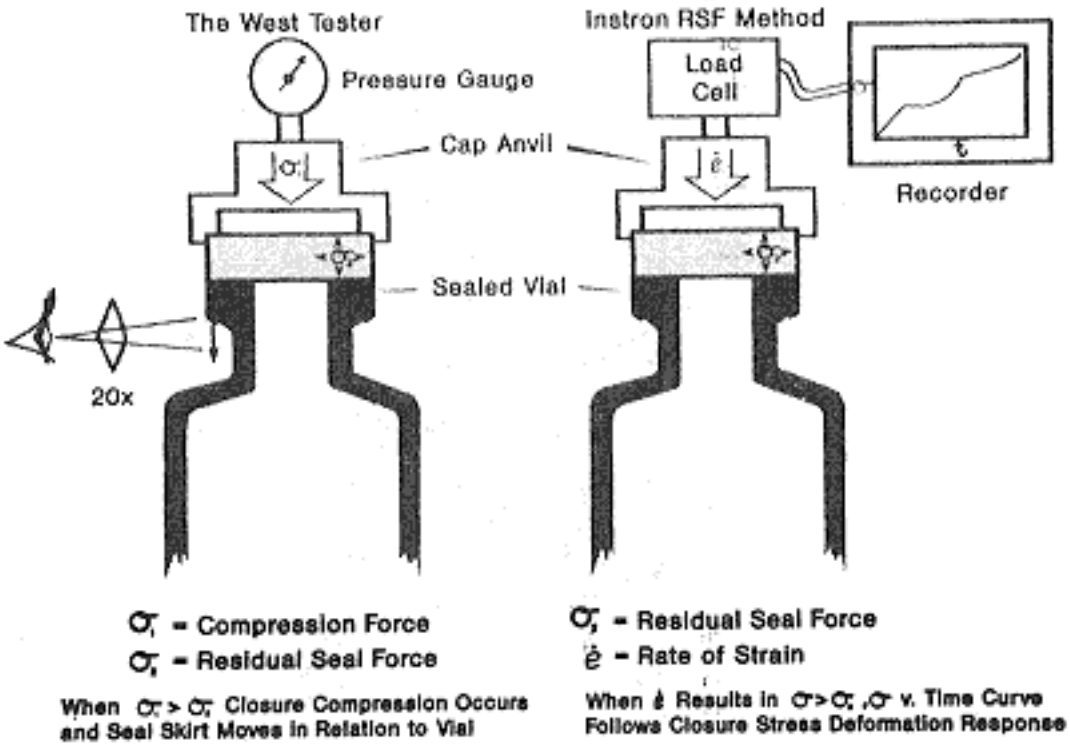


Fig. 4.22
Residual seal force testing methodsthe West seal force tester vs. the constant rate of strain seal force tester (courtesy of the Parenteral Drug Association, Inc., Bethesda, Maryland). Source: Ref. 27.

Package Shipping and Storage

The distribution environment challenges of temperature, pressure, humidity fluctuations, and shock and vibration should be considered in evaluating containers for integrity. To test these effects 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 (30). 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

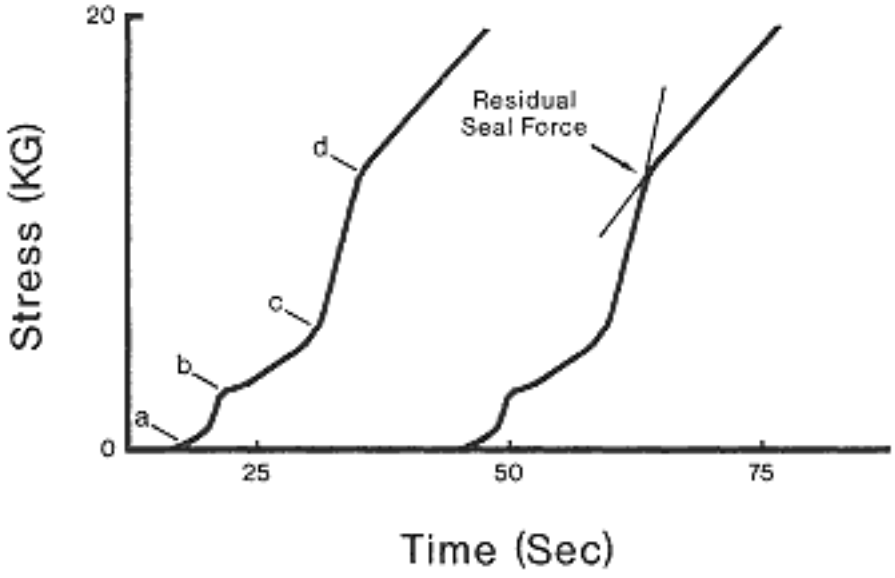


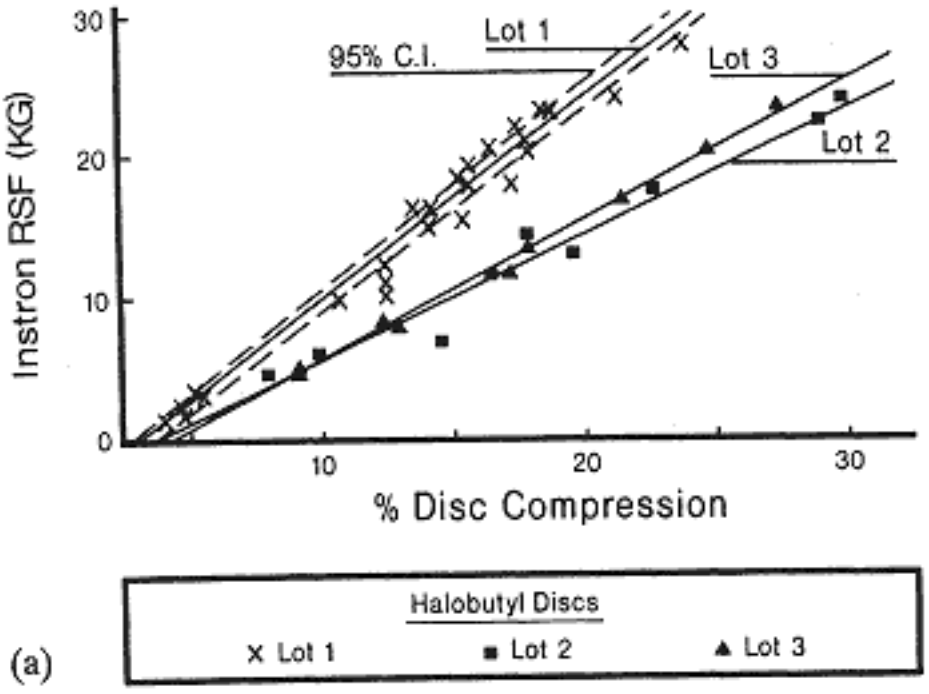
Fig. 4.23
Instron stress deformation response curve (courtesy of the Parenteral Drug Association, Inc., Bethesda, Maryland).
Source: Ref. 29.

specific integrity test failures, simulated shipping studies can be performed in the laboratory.

Aged product will often exhibit different package integrity than when first manufactured, often due to aging of polymeric packaging materials or the relaxation of viscoelastic packaging materials. This is exemplified by the decay in residual seal force of a stoppered vial or the drop in removal torque of a screw-capped bottle. Therefore, package integrity validation is not complete until the effects of time 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.

Conclusion

In conclusion, package integrity is a simple concept but one that is not easily measured or validated. 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



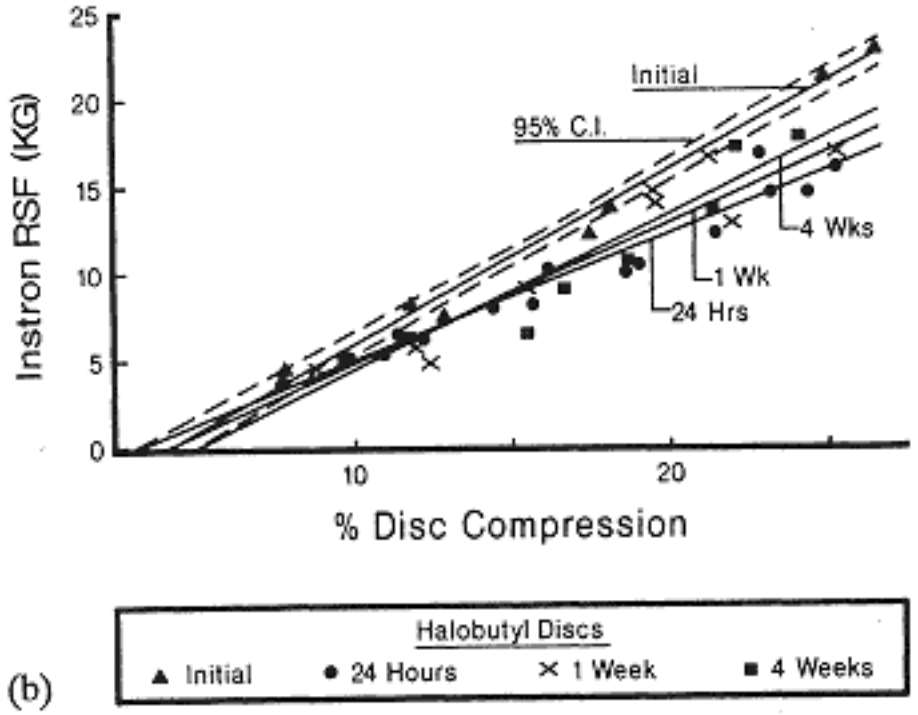
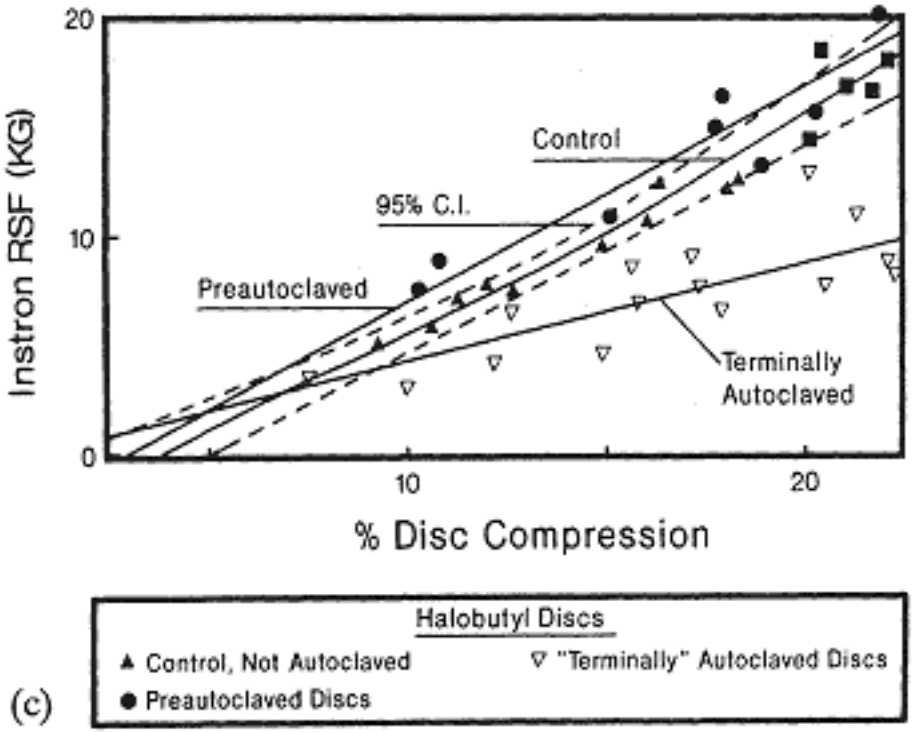


Fig. 4.24
Residual Seal Force (RSF) test results (courtesy of the *Journal of Parenteral Science and Technology*). (a) RSF closure lot to lot differences; (b) time-after-capping effects on RSF; (c) terminal steam sterilization effects on RSF. Source: Ref. 29.



(c)

the same way that nothing is absolutely clean or pure, all 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 must be weighed against other factors such as reliability, speed, cost, ease of use, repeatability, safety, and data processing capabilities.

The pharmaceutical industry has historically taken a liberal approach to package integrity validation. If nothing appeared to leak through the seal, if the samples tested were sterile, and if product stability was satisfactory, package integrity was considered validated. This approach has been found to be insufficient due to a variety of

factors; including today's more stringent regulatory environment, the rise in complex parenteral packaging and product delivery devices, and the growing number of products sensitive to even minute leakage of gases or moisture.

To meet these challenges a more comprehensive product/package development approach is recommended. This begins by precisely defining the functions and expectations of the package in terms of integrity, stability, and functional performance. Then package materials and component designs can be selected; followed by the optimization of package filling, assembly, and processing operations. Wherever possible, the integrity and functional performance of the package should be challenged at the limits of component dimensional specifications and filling, assembly, and processing parameters. Checks must be integrated into each critical step to monitor and ensure adequate packaging and process controls. Finished product must also be evaluated as a function of the distribution and long-term storage environment. Once product development is complete, continual monitoring of critical points of the operation serve as the primary tool for assuring package integrity. Leakage tests of the finished product should only be considered a final check of 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) mediumsterile 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 32°C

5. One incubator at room temperature

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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, 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, ampuls, 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.

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	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 ampuls, 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 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.

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	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 32°C and TSB at 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 sterility test filter holder unitMillipore Sterifil, Falcon unit

2. Membrane filter, 0.45 mm, 47 mm, hydrophobic edge

3. Sterile needles, syringes, or administration sets20

4. Sterile trypticase soy broth (TSB), 100 ml tubes3

5. Sterile fluid thioglycollate medium (FTM), 100 ml tubes3

6. Sterile diluting fluid, 100 ml3

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7. Sterile scissors

8. Sterile forceps, smooth tip, 4½6 in. 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 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, ampuls, 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.

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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. Remove overseals from the necks of test samples, previously disinfected ampuls, multidose vials or large-volume containers. Wipe the rubber diaphragm or neck of ampuls with 70% alcohol.	Do not leave excess alcohol on closure.
7. For ampuls, 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 source. 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 ampuls or vials. Insert needle through rubber closure of vials or into opened ampuls 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.

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	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 610 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 of 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.

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	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 three and seven days for presence of microbial growth.	Incubate FTM at 32°C and TSB at 25°C (RT). Longer incubation may be required at times to permit slow growers to develop (e.g., 1014 days).
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
Example of Methods Used to Clean, Assemble, Sterilize, and Make Final Connections with Membrane Filtration Equipment

Cleaning

1. Mainfold: After testing has been completed for the day, flush completely with hot water followed by distilled water, then drain thoroughly and allow to dry overnight.

2. Filter holders: Clean after each use by using a mechanical dishwasher with an extended five-minute wash cycle or by immersing in a detergent solution. After a soaking period in the detergent solution, rinse in hot tap water and distilled water, backflushing the fritted-glass bases to dislodge any trapped material. Parts may be wiped dry with

lint-free toweling. If holders are to be sterilized the same day, dry the fritted-glass base with lab air or vacuum.

3. Swinney Adapter: After use, soak in alcohol, detergent, or other suitable agent. Disassemble and remove used prefilters from wire screen, dry, and reassemble with clean prefilters.

Assembly.

1. Slide a rubber stopper over the outlet tube of each filter holder base and place each base in its manifold support. Place a funnel on each base and clamp in place with a spring-action clamp.

2. Insert a host adapter into each rubber funnel cover and attach a three-way stopcock to the adapter. A similar adapter in the end of the rubber tubing at each manifold position is attached to the opposite end of the three-way stopcock.

3. On the side arm of the stopcock, place a Swinney Hypodermic Adapter containing a single 13-mm diameter Microfiber Glass Prefilter.

4. Place two 13-mm diameter Microfiber Glass Prefilters in each of the remaining six Swinney Hypodermic Adapters and attach each adapter to its fitting on the side of the manifold valves.

5. Wrap the ends of the manifold openings with paper for sterilization.

Sterilization

1. Open all manifold stopcocks. Failure to open stopcocks may result in damage to the filters and inadequate sterilization. Residual moisture could also damage the filters. Steam sterilize the entire assembly.

Final Connection

1. When the assembly is cool, move it to a sterile area and close all stopcocks. Connect the vacuum tubing to a large receiver (water trap) beneath the bench and connect the receiver to a vacuum source.

2. Flame and connect flushing manifold to a pressurized source of sterile diluting fluid.

3. Aseptically place a sterile membrane filter onto each of the filter funnel units on the sterility test unit. The apparatus is now ready to use.

4. Extra filter funnel units, with membrane filters in place, may be wrapped individually and autoclaved. These can be put in place on the sterility test unit and used for testing after the original units have been used.

Appendix IV
Aseptic Procedures at the Laminar Flow Workbench

Note: The laminar flow 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

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clean, non-linting sponge dampened with distilled water. The entire inside of the workbench should then be wiped with another clean, non-linting 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 non-use, 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 antisepteic. Each must then don a clean cap that provides complete coverage of head hair and a clean, non-linting, 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 in order 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 non-sterile 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 six 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 ampuls should be cleaned, preferably with 70% alcohol and a non-linting sponge, prior to the introduction of the needle for removal or addition of drugs.

12. Avoid the spraying of 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. Where 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 1012 p.s.i.g.). There are at least two disadvantages of the vacuum system as compared with the pressure system: (1) any leakage draws contamination into the container and system; (2) 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 tamper-proof 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 re-entry into the workbench.

Appendix V
Comparison of Various LAL Methods

Courtesy of Thomas J. Novitsky, Ph.D., Associates of Cape Cod, Inc., Woods Hole, MA, and *LAL Update*, published by Associates of Cape Cod, Inc.

Method	Reagents needed	Equipment needed	Endpoint
Gel-clot	LAL	37°C water or dry bath	Gel-clot
Turbidimetric	LAL	37°C water or dry bath or oven & spectro-photometer	Turbidity
Turbidimetric end point	LAL	Spectrophotometer & 37°C water bath or dry bath	Turbidity
Chromogenic automated microtiter	LAL & chromo-genic substrate, tris buffer & acid	Cetus pro/pette system, microplate reader & 37°C incubator block	Yellow color
Turbidimetric kinetic auto-matic gelclot	LAL	Toxinometer	Turbidity or gel-clot
Turbidimetric kinetic	LAL	Spectrophotometer with kinetic analyzer & temp. controller	Turbidity
Turbidimetric kinetica	LAL	LAL-4000	Turbidity
Chromogenicb	LAL & acid & chromogenic reagent & buffer	37°C water or dry bath or oven & spectrophotometer	Yellow color
Microassay chromogenic pseudo-kinetic	LAL & chromo-genic substrate & MgCl2 solution	Microplate Microplate 37°C Incubator	Yellow color
Chromogenic HPLC	LAL & chromo-genic substrate, tris buffer & acid	HPLC or spectrophotom-eter, 37°C water bath, ice bath & membrane filter	Yellow color

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Method	Incubation time	Skill level	Sensitivity	Cost per test	Total operator timeReference
Gel-clot	60 min	Low	0.021.0 EU/ml	Low	60 min(1,2)
Turbidimetric	3060 min	Moderate to high	0.011.0 EU/ml	Moderate	60 min(3,4)
Turbidimetric end point	60 min	High	0.0011 ng/mL	Moderate	80 min(5)
Chromogenic automated microtiter	22 min	Low	0.010.12 EU/mL	Moderate	3045 min(6)
Turbidimetric kinetic auto-matic gelclot	10100 min	Low	0.0005500 EU/mL	Moderate	20110 min(7)
Turbidimetric kinetic	20 min	Moderate	0.01100 ng/mL	Moderate	40 min(8)
Turbidimetric kinetica	1090 min	Low	0.001100 EU/mL	Moderate	20100 min(9,10)
Chromogenicb	1020 min	High	0.050.5 EU/ml	Moderate	60 min(11,12)
Microassay chromogenic pseudo-kinetic	6180 min	High	0.0050.2 ng/mL	Moderate	7090 min (13)
Chromogenic HPLC	4060 min	High	0.51.5 ng/mL	Moderate	80120 min(14)

Method	Reagents needed	Equipment needed	Endpoint
Colorimetric	LAL & Lowry protein reagent	37°C water or dry bath or oven & spectro-photometer & centrifuge	Blue color
Nephelometric	LAL	37°C water or dry bath or oven & nephelometer	Turbidity
Kinetic	LAL	Abbott MS-2	Turbidity
Kinetic	LAL	Modified microplate reader	Turbidity
Slide (gel-clot)	LAL	37°C oven & microscope slide	Gel-clot
Slide (dry-up)	LAL	37°C oven & microscope slide	Dry-clot
Slide (wells)	LAL & dye & petrolatum	37°C oven & microscope slide	Gel-clot
Slide (capillary)	LAL	37°C oven & microscope slide & capillary tube	Gel-clot
Slide (stain)	LAL & dye	37°C oven & microscope slide	Gel-clot
Slide (phase contrast)	LAL	37°C oven & microscope slide & microscope	Gel-clot
Micromethod	LAL	37°C oven & microplate & capillary tube	Gel-clot
Micromethod	LAL	Capillary tubes & hydro-static pressure, device & 3040°C incubator	Gel-clot

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Method	Incubation time	Skill level	Sensitivity	Cost per test	Total operator timeReference
Colorimetric	1020 min	High	0.050.5 EU/ml	Moderate	60 min(15)
Nephelometric	3060 min	Moderate to high	0.011.0 EU/ml	Moderate	60 min(16)
Kinetic	2090 min	Low	0.0005500 EU/ml	High	20 min(17,18)
Kinetic	2090 min	High	0.005500 EU/ml	High	20 min(19)
Slide (gel-clot)	3045 min	Moderate	0.021.0 EU/ml	Low	60 min(20,21)
Slide (dry-up)	30 min	Moderate	0.52.5 EU/ml	Low	60 min(22)
Slide (wells)	30 min	Moderate	0.151.0 EU/ml	Low	75 min(23)
Slide (capillary)	3045 min	Moderate	0.021.0 EU/ml	Low	60 min(24)
Slide (stain)	30 min	Moderate	5.0 EU/ml	Low	60 min(25)
Slide (phase contrast)	3045 min	Moderate	0.021.0 EU/ml	Low	75 min(25)
Micromethod	60 min	Moderate	0.301.0 EU/ml	Low	60 min(26)
Micromethod	1060 min	Moderate	1 ng/mL	Low	70 min(27)

Method	Reagents needed	Equipment needed	Endpoint
Microtechnique	LAL	37°C oven & tube of dye & capillary tube	Gel-clot
Microdilution	LAL & dye	37°C oven & microplate	Gel-clot
LAL-bead	LAL	37°C water or dry bath & beads & tray & rocker platform	Gel-clot
Radioisotope	LAL & 1251-labeled coagulogen	37°C water or dry bath & centrifuge & gamma counter	Gel-clot
Rocket	LAL & anticoagulogen antibody	37°C water or dry bath & gel electrophoresis	Gel-clot

aA newer Turbidimetric Kinetic method is now available utilizing the LAL-5000, the LAL-5000 Series II and an incubating microplate reader. See *LAL Update* Vol 5 (3); Vol. 9 (1), and Vol 10 (3).

bA relatively new single step assay method is described by Lindsay GK, Roslansky PF, and Novitsky TJ, Single-Step, Chromogenic Limulus Amebocyte Lysate Assay for Endotoxin, *J. Clin. Micro., 27*, 947951 (1989).

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Method	Incubation time	Skill level	Sensitivity	Cost per test	Total operator timeReference
Microtechnique	45 min	Moderate	0.051.0 EU/ml	Low	75 min(28)
Microdilution	60 min	Moderate	0.301.0 EU/ml	Moderate	60 min(29)
LAL-bead	90 min	Moderate to high	5.0 EU/ml	Moderate	90 min(30)
Radioisotope	4050 min	High	0.050.6 EU/ml	High	120 min(31)
Rocket	60 min	High	0.05 EU/ml	High	3 hr(32)

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Appendix VI
Proposed Changes for the USP XXII Section on Particulate Matter in Injections

The entire USP XXII section on Particulate Matter in Injections is to be revised according to the MarchApril, 1993 issue of *Pharmacopeial Forum (PF)* (pp. 49394956; reprinted with permission of the United States Pharmacopeial Convention, Inc. in this Appendix). The revision resulted from a report by the Home and Hospital Parenteral Products Subcommittee of the Health Industry Manufacturers Association (HIMA). This report appeared in the MayJune, 1993 issue of *PF*, Stimuli to the Revision Process, Final Report: Improved Microscopic Assay for the Enumeration of Particulate Matter in Parenteral Solutions. The most significant changes to the USP particulate matter test requirements according to this proposal include the following:

1. Light obscuration methods will be the primary method for the testing of particulate matter in both Large Volume Injections (LVIs) and

Small Volume Injections (SVIs). It is expected that most products, regardless of volume, will conform to USP requirements based on light obscuration alone. USP XXII currently requires LVIs to be examined for particulate matter by microscopic methods (see pp. 206211).

2. Microscopy will be used as a secondary or back-up particle test method for both LVIs and SVIs. Microscopy will be required only when necessary to reach a conclusion on conformance to particulate requirements when light obscuration methods are inconclusive.

3. Details on both methodstest apparatus, standardization, testing environment, and testing procedureshave been significantly expanded and strengthened.

4. Light obscuration calculations are provided not only for pooled samples (SVI), but also for individual samples (SVI) and individual unit samples (LVI).

5. Of greatest significance, the proposed acceptance criteria have been greatly tightened. The proposed new specifications are compared to the current specifications in the table below.

	Particles ≥ 10 µm			Particles ≥ 25 µm		
		Proposed			Proposed	
Type of Container/Product	Current	LO	Micro	Current	LO	Micro
Small Volume Injections (Particles Per Container)						
Powders	10,000	5,000	2,500	1,000	500	250
Liquids	10,000	2,000	1,000	1,000	200	100
Large Volume Injections (Particles Per Millimeter)	50	25	12	25	3	2

Note the following proposed changes:

For SVI powders, the proposed limits by light obscuration methods are one-half the current USP XXII limits.

For SVI liquids, the proposed limits by light obscuration methods are one-fifth the current USP XXII limits.

For SVI powders, the proposed limits by microscopic methods are one-fourth the current USP XXII limits.

For SVI liquids, the proposed limits by microscopic methods are one-tenth the current USP XXII limits.

For LVIs, the proposed limits have been greatly reduced, particularly at the 25 µm size.

These proposed changes in particulate matter testing and specifications will be the subject of extensive controversy and debate over the next several years both before and after these changes become official in future USP editions.

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<788> Particulate Matter in Injections, *USP XXII* page 1596 and page 3145 of the *Seventh Supplement*. The proposed revisions to reorganize this general chapter are in part based on data and text provided to the Home and Hospital Parenteral Products (HHP) Subcommittee in a report by the Health Industry Manufacturers Association (HIMA), which will appear in *PF* 19(3) [MayJune 1993] as a *Stimuli to the Revision Process entitled Final Report: Improved Microscopic Assay for the Enumeration of Particulate Matter in Parenteral Solutions*. An example of the filtration funnel used in the *Microscopic Particle Count Assay* is the Gelman #4203 25-mm polysulfone filtration apparatus with 200-mL twist lock funnel. For the filtration diffuser in this same assay, a Millipore® stainless support screen, catalog #XX30 025 10, can be used. For the petri dish the Millipore® petrislide or similar propietary device can be used.

HIMA's report focused on large-volume injections and gave strong support for a new approach to evaluation of injections. Total solid and nonsolid particles are determined by a light obscuration measurement, and this rapid method is expected to be sufficient for most demonstrations of compliance. The next stage is to measure solid particles only, using the improved microscopic assay. Both methods are presented here for comment. Note that in addition to the new microscopic method, the test by light obscuration also has been extensively reworked and updated.

The HHP Subcommittee finds that this two-measurement basis of particulate matter limits is more meaningful than the present simple method standards for large and small (light obscuration) injections, so the Subcommittee is extending the two-measurement approach also to small-volume injections. This effort has been in conjunction with a separate series of studies under the auspices of the Quality Control Section of the Pharmaceutical Manufacturers Association.

Particulate matter methods and limits eventually will be of interest to international harmonization. The establishment of explicit method(s) of measurement is the key issue in this regard, as this relates to laboratory capital goods and analyst training. The Subcommittee believes that the light obscuration method has, in some variant or other, wide usage, so no concern arises. In contrast, the improved microscopic assay is new both here and abroad. The Subcommittee expects that a period of time will elapse before other regional, or national, pharmacopeias' scientists have evaluated the

method and decided whether it is necessary in their procedures. For either method of measurement, the actual limits specified also may differ among pharmacopeias, but this causes less harmonic distortion than is caused by the purchase of different equipment and divergent training. Left standing is the issue of the scope of application of any limit test(s). Many major market areas will likely require large-volume injections limits. USP also sets limits for many small-volume injections.

An Open Conference is planned for July in Colorado Springs, Colorado. It will offer opportunities for group discussion of these proposed changes to the general chapter. Contact Anju Malhotra, Drug Standards Division, for details.

3PO2800 (HHP) RTS1175501

<788> Particulate Matter in Injections

Change to read:

▲Particulate matter consists of mobile, randomly-sourced, extraneous substances, other than gas bubbles, that cannot be quantitated by chemical analysis due to the small amount of material that it represents and to its heterogeneous composition. Injectable solutions, including solutions constituted from sterile solids intended for parenteral use, should be essentially free from particles that can be observed on visual inspection. The tests described herein are physical assays performed for the purpose of enumerating subvisible extraneous particles within specific size ranges.

Procedures for the determination of particulate matter based on microscopy and light obscuration counting are given herein, to be applied in accordance with

specified test plans for specific product types. It is expected that most articles will conform based on light obscuration alone. Testing, however, at both stageslight obscuration followed by microscopymay be required to reach a conclusion on conformance to requirements.

All large-volume injections (LVI) for single-dose infusion, and those small-volume injections (SVI) for which the monographs specify such requirements, are subject to the particulate matter limits set forth for the test being applied. Individual monographs may establish other limits than those given herein.

Not all injection formulations can be examined for particles by one or both of these tests. Emulsions, colloids, and liposomal preparations are examples. Refer to the specific monographs when a question of test applicability occurs. Higher limits are appropriate for certain articles and are specified in the individual monographs.

In the tests described below for large-volume and small-volume injections, the results obtained in examining a discrete unit or group of units for particulate matter cannot be extrapolated with certainty to other units that remain untested. Thus, statistically sound sampling plans based upon known operational factors must be developed if valid inferences are to be drawn

from observed data to characterize the level of particulate matter in a large group of units. Sampling plans should be based on consideration of product volume, numbers of particles historically found to be present in comparison to limits, particle size distribution of particles present, and variability of particle counts between units.

LIGHT OBSCURATION PARTICLE COUNT ASSAY

The test applies to LVI labeled to contain greater than 100 mL in volume, except where excluded by monograph requirement. It counts suspended particles that are solid or liquid. This test also applies to all single-dose or multiple-dose SVI in containers that are labeled as containing 100 mL or less that are either in solution or in solution constituted from sterile solids, wherever a requirement for a limit for particulate matter appears in the individual monograph. Injections packaged in prefilled syringes and cartridges are exempt from these requirements, as are products for which an individual monograph specifies that the label states that the product is to be used with a final filter.

Test Apparatus

The test calls for the use of an electronic liquid-borne particle counter system utilizing a white light,

laser diode, or gas laser light-obscuration sensor with a suitable sample feeding device. Ascertain that the operating parameters of the instrumentation applied are appropriate to the required accuracy and precision of the test result and that adequate training for those technically responsible for the test is provided. The apparatus qualification given here defines the principles to be followed to ensure instruments meet general performance criteria and accuracy appropriate to analyses of particles in injectable materials. Critical operational criteria consist of the following.

*Sensor concentration limits*The instrument applied shall have a concentration limit (the maximum number of particles per mL) identified by the manufacturer that is greater than the concentration of particles in the sample to be counted. The vendor-certified concentration limits for a sensor shall be specified as that count level at which coincidence counts, due to simultaneous presence of two or more particles in the sensor view volume and due to electronic or sensor view volume spatial considerations, comprise less than 5% of the count events collected.

*Sensor dynamic range*The dynamic range of the instrument used (range of particle sizes which can be accurately sized and counted) must include the smallest particle size to be enumerated in the test articles.

*Sensor illumination*Light obscuration sensors with white light, laser diode, or gas laser light sources may be applied. Specific calibration methods may be required for individual types of sensors as indicated in the appropriate section below or defined in the manufacturer's instructions.

Instrument Standardization

The following information for instrument standardization helps ensure that the sample volume accuracy, sample flow rate, particle size response curve, sensor resolution, and count accuracy are appropriate to performance of the test. The user should verify that, whenever used, the counting unit and bottle sampler are functioning to vendor specifications.

SAMPLE VOLUME ACCURACY

Since the particle count from a sample aliquot varies directly with the volume of fluid sampled, it is important that the sampling accuracy is known to be within a certain range. For a sample volume determination, fill all of the dead (tare) volume in the sample feeder with *Water for Injection* or distilled water that has been passed through a filter with a retention rating not exceeding 1.2 µm: transfer 10 mL of the *Water for Injection* to a tared container, withdraw 5 mL through the sample feeding device, and again weigh

the container. Determine the sample volume by subtracting the tare volume from the combined sample plus tare volumes. Verify that the value obtained is within ±5% of the appropriate sample volume for the test. Alternatively, the sample volume may be determined using a Class A 10-mL graduated cylinder (see *Volumetric Apparatus* <31>). [NOTEinstruments of this type require a variable tare volume. This is the amount of sample drawn prior to counting. This volume may be determined for syringe-operated samplers by setting the sample volume to zero and initiating sampling, so that the only volume of solution drawn is the tare. The tare volume is subtracted from the total volume of solution drawn in the sampling cycle to determine the sample volume.]

SAMPLE FLOW RATE

Verify that the flow rate is within the manufacturer's specifications for the sensor used. This may be performed using a calibrated stop watch and measuring the time required for the instrument to draw and count a specific sample volume (i. e., the time between beginning and ending of the count cycle as denoted by instrument indicator lights or other means).

CALIBRATION

Manual MethodCalibrate the instrument with a minimum of 3 calibrators, each consisting of near-

monosize polystyrene spheres, approximately 10-, 20-, and 30-µm diameters, in an aqueous vehicle.1 Laser diode sensors require additional calibration spheres at 15-µm diameter. The number of spheres counted must be within the sensor's concentration limit. Prepare suspensions of the calibrator spheres in water at a concentration of 1000 to 5000 particles per mL, determine the channel setting that corresponds to the highest count setting for the sphere distribution. This is determined by using the highest count threshold setting to split the distribution into two bins containing equal numbers of counts, with the instrument set in the differential count mode (moving window half-count method). Use only the central portion of the distribution in this calculation to avoid including asymmetrical portions of the peak. The portion of the distribution, which must be divided equally, is the count window. The window is bounded by threshold settings that will define a threshold voltage window of ±20% around the mean diameter of the test spheres. The window is intended to include all single spheres, taking into account the standard deviation of the spheres and the sensor resolution, while excluding noise and ag-

1ASTM standards F322-80 and F658-87 provide useful discussions pertaining to calibration procedures applying near-monosize latex spheres.

gregates of spheres. The value of 20% was chosen based on the worst-case sensor resolution of 10% and the worst-case standard deviation of the spheres of 10%. Since the thresholds are proportional to the area of the spheres rather than the diameter, the lower and upper voltage settings are determined by the formula:

$$VL = Vs\ (0.64),$$

in which *VL* is the lower voltage setting and *Vs* is the voltage at the peak center, and

$$VU = Vs\ (1.44),$$

in which *VU* is the upper voltage setting.

Once the center peak thresholds are determined, use these thresholds for the standards to create a regression of log voltage versus log particle size, from which the instrument settings for the 10- and 25-µm sizes can be determined. If particles smaller than 10 µm are to be enumerated, calibration standards must be run that are less than or equal to the smallest size to be counted.

Automated MethodThe calibration (size response) curve may be determined for the instrument-sensor system using validated software routines offered by instrument vendors; these may be included as part of the instrument software or used in conjunction with a

microcomputer interfaced to the counter. The use of these automated methods is appropriate if the vendor supplies written certification that the software provides a response curve equivalent to that attained using the manual method and if the automated calibration is validated as necessary by the user.

Electronic MethodUsing a multi-channel peak height analyzer (MCA), determine the center channel of the particle counter pulse response for each standard suspension. This peak voltage setting becomes the threshold used for calculation of the voltage response curve for the instrument. The standard suspensions to be used for the calibration are run in order, and median pulse voltages for each are determined. These thresholds are then used to generate the size response curve manually or via software routines. The thresholds determined using the MCA data are then transferred to the counter to complete the calibration. If this procedure is used with a comparator-based instrument, the comparators of the counter must be accurately adjusted beforehand.

SENSOR RESOLUTION

The particle size resolution of the instrumental particle counter is dependent upon the sensor used and may vary with individual sensors of the same model.

Determine the resolution of the particle counter for 10-µm particles using the monosized 10-µm calibrator spheres. The relative standard deviation of the count must be less than 5%. Two acceptable methods of determining particle size resolution are (1) manually determining the amount of peak broadening due to instrument response; and (2) using an electronic method of measuring and sorting particle sensor voltage output with a multichannel analyzer.

Manual MethodAdjust the particle counter to operate in the cumulative mode or total count mode. Refer to the calibration curve obtained earlier and determine the threshold voltage for the 10-µm monosize spheres. Adjust 3 channels of the counter to be used in the calibration procedure as follows:

Channel 1 is set for 90% of the threshold voltage.

Channel 2 is set for the threshold voltage.

Channel 3 is set for 110% of the threshold voltage.

Draw a sample through the sensor, observing the count in Channel 2. When the particle count in that channel has reached approximately 1000, stop counting and observe the counts in Channels 1 and 3. Check to see if the Channel 1 count and the Channel 3 count are 1.68 and 0.32 times the count in Channel 2, ± 10%, respectively. If not, adjust Channel 1 and Channel 3 thresholds to meet these criteria. When these criteria

have been satisfied, draw a sample of suspension through the counter until the counts in Channel 2 have reached approximately 10,000, or until 10 mL of the sphere suspension has been counted. Verify that Channel 1 and Channel 3 counts are 1.68 and 0.32 times the count in Channel 2, ± 3%, respectively.

Record the particle size for the thresholds just determined for Channels 1, 2, and 3. Subtract the particle size for Channel 2 from the size for Channel 3. Subtract the particle size for Channel 1 from Channel 2. The values determined are the observed standard deviations on the positive and negative side of the mean count for the 10-µm standard. Calculate the resolution of the sensor taken by the formula:

$$\frac{100\sqrt{S_o^2 - S_s^2}}{D}$$

in which *So* is the highest observed standard deviation determined for the sphere, *Ss* is the supplier's reported standard deviation for the spheres, and *D* is the diameter, in microns, of spheres as specified by the supplier. The resolution is not more than 10%.

Automated MethodSoftware is available for some counters that allows for the automated determination of sensor resolution. This software may be included in the instrument or used in conjunction with a mi-

crocomputer interfaced to the counter. The use of these automated methods is appropriate if the vendor supplies written certification that the software provides a resolution determination equivalent to the manual method and if the automated resolution determination is validated as necessary by the user.

Electronic MethodRecord the voltage output distribution of the particle sensor, using a multi-channel analyzer (MCA) while sampling a suspension of the 10 µm particle size standard. To determine resolution, move the MCA cursor up and down the electric potential scale from the median pulse voltage to identify a channel on either side of the 10-µm peak that both have approximately 61 % of the counts observed in the center channel. Use of the counter size response curve to convert the mV values of these two channels to particle sizes provides the particle size at ± 1 standard deviation from the 10-µm standard. These values can then be used to calculate the resolution as described under *Manual Method*.

DETERMINATION OF PARTICLE COUNTING ACCURACY

System SuitabilityPrepare the suspension and blank from the *USP Particle Count Reference Standard* in the following sequence. Remove outer closures, sealing bands, and any loose or shedding paper

labels and wash the exterior of containers using the following procedure. Cleanse glassware, closures, and other required equipment by immersing and scrubbing in warm, nonionic detergent solution, then rinsing in flowing warm tap water, followed by rinsing in flowing filtered water. Organic solvents may be used to facilitate cleaning. Finally, pressure-rinse in filtered water, using a hand-held pressure nozzle with final filter, or other appropriate equipment. Dry in a particle-free airflow.

DeterminationSet the instrument to count at 10 and 15 µm. Mix by inverting the suspension 25 times within 10 seconds. Degas by mild ultrasonication for 30 seconds or by allowing to stand for 2 minutes. Remove the closure. Gently stir contents of containers by hand swirling or by mechanical means, taking care not to introduce air bubbles or contamination. Stir continuously throughout the analysis. Sampling directly from the container, take 3 consecutive portions, each not less than 5 mL. Discard the data from the first portion. [NOTEComplete the determination within five minutes.]

Repeat this same procedure using the blank in place of the suspension.

CalculationsAverage the counts resulting from the analysis of the 2 portions of the suspension at 10 µm.

Average the results from the analysis of the 2 portions of blank at 10 µm. Calculate the number of particles in each mL by the equation:

$$(Ps - Pb)/V,$$

in which *Ps*, is the average particle count obtained from the suspension, *Pb* is the average particle count obtained from the blank, and *V* is the average volume, in mL, of the 4 portions tested. Repeat the calculations, using the results obtained at 15 µm.

InterpretationThe instrument meets the suitability test for Determination of Particle Counting Accuracy if the count obtained at 10 µm is between 3250 and 4250 per mL, and the ratio of the counts obtained at 10 µm to those obtained at 15 µm is between 1.5 and 3.5.

If the instrument does not meet the test for Determination of Particle Counting Accuracy, recalibrate with the remaining suspension and blank. If the results of the second test are within the limits given above, the instrument meets the Determination of Particle Counting Accuracy test. Should the system not meet the test for Determination of Particle Counting Accuracy on the second attempt, the source of the failures should be determined, corrected, and retested.

When gaining experience with the *System Suita-*

bility test, alternative determination of count accuracy by microscopy is valuable. Using standard spheres of 15-µm to 30-µm nominal diameter, prepare a suspension containing approximately 100 to 200 particles per mL. Perform five 5-mL sample counts on this suspension using the particle counter 10-µm size threshold to obtain a mean total count. Using a pipette, withdraw a volume of this suspension containing 500 to 1000 particles per mL, and transfer to a filter funnel with a membrane filter as described under *Supplies* in the *Microscopic Particle Count Assay*. After drying the membrane, count the total number of standard spheres collected on the membrane filter. This count should agree with the mean per mL instrumental count for the suspension ± 20%.

Test Environment

The test must be performed in an environment that does not contribute any significant amount of particulate matter. Specimens must be cleaned to the extent that any level of extraneous particles added has a negligible effect on the outcome of the test. The specimen, glassware, closures, and other required equipment should be prepared in an environment protected by high-efficiency particulate air (HEPA) filters. Nonshedding garments and nonpowdered gloves should

be worn throughout the preparation of samples.

Cleanse glassware, closures, and other required equipment by immersing and scrubbing in warm, non-ionic detergent solution. Rinse in flowing tap water, and then rinse again in flowing filtered water. Organic solvents may also be used to facilitate cleaning. Finally, rinse the equipment in filtered water using a hand-held pressure nozzle with final filter or other appropriate filtered water source such as distilled water passed through a capsule filter. The filter used should be of 1.2 µm or lower retention rating.

A particulate control (blank) test is conducted to determine that the environment is suitable for the analysis and that the glassware is properly cleaned, and to ensure that the water used for analysis does not contain numbers of particles that might interfere with the test. It also serves to indicate that the counter being used is not generating spurious counts. Using filtered water and cleaned glassware, take 5 consecutive water samples of 5 mL each. Invert each sample 20 times. Degas by sonication for 30 seconds or by allowing to stand for 2 minutes. Stir each water sample by mechanical means at a speed sufficient to maintain a slight vortex throughout the analysis. If 5 particles of 25-µm or 25 particles of 10-µm or greater size are observed for the combined 25 mL, either the environment is not suit-

able for particulate analysis or the filtered water and glassware have not been properly prepared. Repeat the preparatory steps until environment, water, and glassware are suitable for this test.

To collect background counts, use a cleaned vessel of type and volume representative of that to be used in the test. Place a volume of 20 mL of filtered water in the vessel. Agitate the sample in the cleaned glassware by inversion or swirling. Degas by sonication for 30 seconds or by allowing to stand for 2 minutes. Swirl the vessel containing the water sample by hand or agitate mechanically to suspend particles. Withdraw and count 3 consecutive samples of not less than 5 mL each, disregarding the first count. If more than 10 particles of 10 µm or greater size, or more than 2 particles of 25 µm or greater size are observed in the combined 10-mL sample, the environment is not suitable for particulate analysis: the filtered water and glassware have not been properly prepared or the counter is generating spurious counts. In this case, repeat the preparatory steps until conditions of analysis are suitable for the test.

Test Procedure

TEST PREPARATION

For containers of less than 25-mL in volume, test a solution pool of 10 or more units. Single, SVI units

may be sampled if the individual unit volume is 25 mL or greater. For LVI, the entire contents of single units are tested.

Prepare the test specimens in the following sequence. Remove outer closures, sealing bands, and any loose or shedding paper labels. Rinse the exterior of containers with filtered distilled water as described under *Test Environment*, and dry under protection from environmental contamination. Withdraw the contents of the containers in the normal or customary manner of use, or as instructed in the package labeling, except that containers with removable stoppers may be sampled directly by removing the closure, or if samples are being pooled, by removing the closure and emptying the contents into a clean container.

DETERMINATION

Liquid Fill (contents of each unit are less than 25 mL)Mix (resuspend) each unit by inverting it 20 times. [NOTEBecause of the small volume of some products, it may be necessary to agitate the solution more vigorously in order to suspend the particles completely and homogeneously.] In a cleaned container, open and combine together the contents of 10 or more containers to obtain a minimum volume of not less than 20 mL. Degas by sonication for 30 seconds or

by allowing to stand for 2 minutes to allow air bubbles to leave the solution. Gently stir the contents of the container by hand swirling or by mechanical means, taking care not to introduce air bubbles or contamination. Withdraw a minimum of 3 sample aliquots into the light obscuration counter sensor, each not less than 5 mL in volume. Discard the data from the first portion.

Liquid Fill (contents of each unit are more than 25 mL)Mix the unit by inverting it 20 times. Degas the solution by sonication or by allowing it to sit undisturbed a sufficient time to allow air bubbles to leave the solution. Remove the closure and insert the counter probe into the center space of the solution. Gently agitate the contents of the unit by hand-swirling or by mechanical means. Withdraw a minimum of 3 sample aliquots into the light obscuration counter sensor, each not less than 5 mL in volume. Discard the data from the first aliquot.

Dry or Lyophilized FillOpen the container, taking care not to contaminate the opening or cover. Constitute with a suitable volume of filtered water, or with the appropriate filtered diluent if water is not suitable. Replace the closure and manually agitate the container sufficiently to dissolve the drug powder. Allow the reconstituted samples to sit for approximately 5

minutes. Prior to analysis, gently stir the contents of the containers by hand-swirling or by mechanical means, taking care not to introduce air bubbles or contamination. Pool the appropriate number of units, and withdraw a minimum of 3 sample aliquots into the light obscuration counter sensor, each not less than 5 mL in volume. Discard the data from the first aliquot.

Solution PoolsFor products packaged in containers that are constructed to hold the drug product and a solvent in separate compartments, mix each unit as directed in the labeling, activating and agitating each unit so as to ensure thorough mixing of the separate components. Analyze the solutions as described under *Test Preparation*.

Multiple-dose ContainersFor products labeled as Pharmacy Bulk Package not for direct infusion, proceed as directed for each unit under *Test Preparation* performing the test on sample aliquots that are equal to the maximum dose given in the labeling. For the calculations below, consider an aliquot to be the equivalent of the contents of one full container.

Calculation of Assay Result

POOLED SAMPLES (SVI)

Average the counts resulting from the 2 or more sample aliquots analyzed. Calculate the number of

particles in each container taken by the equation:

$$PVt/VaN,$$

in which *P* is the average particle count obtained from the portions analyzed, *V*t is the volume of pooled sample, in mL, *V*a is the volume, in mL, of each portion analyzed, and *N* is the number of containers pooled.

INDIVIDUAL SAMPLES (SVI)

Average the counts obtained for the 5 mL or greater sample aliquots from each separate unit analyzed, and calculate the number of particles in each container taken by the equation:

$$PV/Va,$$

in which *P* is the average particle count obtained from the portions analyzed, *V* is the volume, in mL, of the tested unit, and Va is the volume, in mL, of each portion analyzed.

INDIVIDUAL UNIT SAMPLES (LVI)

Average the counts obtained for the two 5-mL or more sample aliquots taken from the solution unit. Calculate the number of particles per mL using the formula:

$$P/V,$$

where *P* is the average particle count for a given 5

mL or greater sample volume, and *V* is the volume of the sample aliquot in mL.

Interpretation

The injection meets the requirements of the test if the numbers of particles present do not exceed the values listed below:

Table 1. Light Obscuration Test Particle Count.*

	≥ 10 µm	≥ 25 µm
Small-volume		
Injections:		
Powders	5000	500 per container
Liquids	2000	200 per container
Large-volume		
Injections:	25	3 per mL

*Numerical results are rounded up to the nearest whole number; values of 0.5 particles or greater are rounded up.

MICROSCOPIC PARTICLE COUNT ASSAY

This section describes a microscopic particulate matter assay, which may be applied to both large-volume or small-volume injections. This test enumerates subvisible, essentially solid, particulate matter

in these products on a per volume or per container basis after collection on a microporous membrane filter. Some articles cannot be tested meaningfully by light obscuration. In such cases individual monographs specify only this microscopic assay. Solutions exempted from analysis using the microscopic assay are identified on a monograph basis. Examples are solutions of viscosity too high to filter readily (e.g., concentrated dextrose, starch solutions, or dextrans). Similarly, products known to contain amorphous, semiliquid or otherwise morphologically indistinct materials should not be tested by the microscopic method. These materials show little or no surface relief and present a gelatinous or film-like appearance. Since in solution this material consists of units on the order of 1 µm or less, which may be counted only after aggregation or deformation on an analytical membrane, interpretation of enumeration may only be suitably performed using an electronic particle counter.

Test Apparatus

MICROSCOPE AND RELATED ACCESSORIES

MicroscopeUse a compound binocular microscope that corrects for changes in interpupillary distance by maintaining a constant tube length. The objective and eyepiece combination of lenses must give

a magnification of 100 ± 10X. The objective must be of 10X nominal magnification, a planar achromat or better in quality, with a minimum numerical aperture of 0.25. In addition, the objective must be compatible with an episcopic illuminator attachment. The eyepieces must be of 10X magnification, with a field number of ≥ 15 (widefield). In addition, one eyepiece must be designed to accept and focus an eyepiece graticule. The microscope must have a mechanical stage capable of holding and traversing the entire filtration area of a 25-mm or 47-mm membrane filter.

IlluminatorsTwo illuminators are required. One is an external, focusable auxiliary illuminator adjustable to give incident oblique illumination at an angle of 10° to 20°. The other is an episcopic brightfield illuminator internal to the microscope. Both illuminators must be of a wattage sufficient to provide a bright, even source of illumination and must be equipped with blue filters.

Circular Diameter GraticuleUse a USP Circular Diameter Graticule matched to the microscope model objective and eyepiece such that the sizing circles are with ± 2% of the stated size at the plane of the stage (see Figure 1).

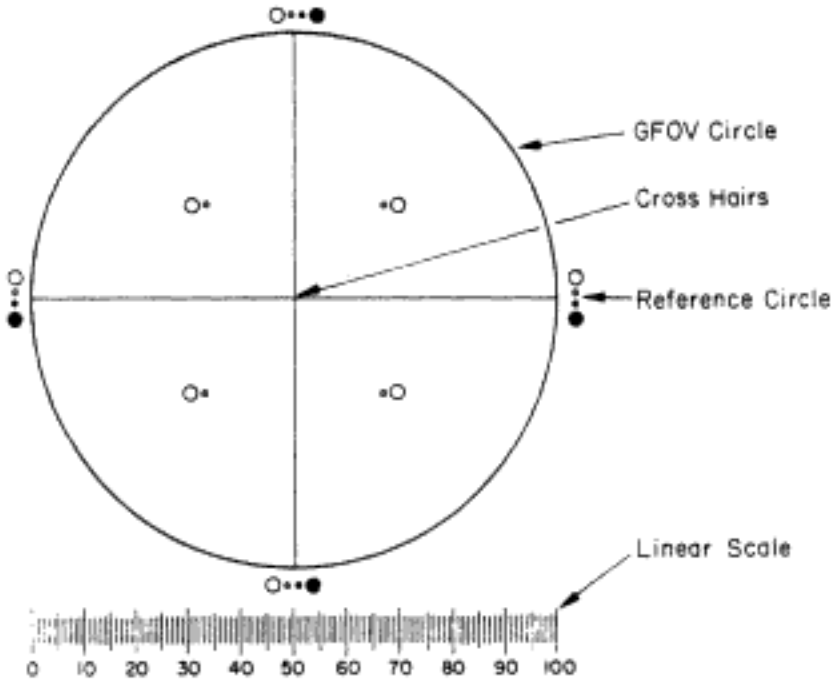

Fig. 1.
USP circular diameter graticule. The large circle divided by crosshairs into quadrants is designated the graticule field of view (GFOV). Transparent and black circles of 10-µm and 25-µm diameter at 100X are provided as comparison scales for particle sizing.

MicrometerUse a NIST certified stage micrometer graduated in 10 µm increments.2

FILTRATION APPARATUS AND SUPPLIES

Filtration FunnelThe funnel used must be capable of holding a volume of ≥ 100 mL and have a minimum effective filtration area of approximately 21 mm in diameter. The funnel may be made of plastic, glass, or stainless steel.

Filtration DiffuserUse a filter support made of stainless steel screen.

2 Suitable supply details to come.

SuppliesUse the following:

A *vacuum source* capable of supplying a vacuum pressure of 46 mm of mercury or greater.

Blunt forceps for handling membrane filters.

A *solvent dispenser* capable of delivering solvents filtered at 1.2 µg or finer retention rating at a range of pressures from 10 psi to 80 psi.

Membrane filters (25 mm or 47 mm nongridded black or dark gray, mixed cellulose ester, with a pore size of 1.0 µm or less).

Petri dish.

Test Environment

A laminar flow hood or other laminar airflow enclosure sufficient to envelope the microscope and area in which the analysis is performed with HEPA-filtered air having not more than 100 particles per cubic foot >0.5 µg in size. For the blank determination, deliver 100 mL of filtered water from the pressure rinser into a filter funnel apparatus set up as described in the preceding section. Apply vacuum and draw the entire volume of water through the membrane filter. Remove the membrane from the filter funnel base and place atop a strip of double-sided tape in a Petrislide or petri dish. After allowing the membrane to dry, examine it microscopically at a magnification of 100X.

If no more than 10 particles ≥ 10 µm in size and no particles ≥ 25 µm or finer are present within the filtration area, the background particle level is sufficiently low for performance of the microscopic assay.

Preparation of Test Apparatus

Throughout this procedure, use suitable nonpowdered gloves and thoroughly clean glassware and equipment that have been rinsed successively with a warm residue-free solution of detergent, hot water, filtered distilled deionized water, and isopropyl alcohol. [NOTEThe distilled deionized water and the isopropyl alcohol should be filtered to a 0.45 µm or lower retention.] Perform the rinsing under the laminar flow enclosure equipped with HEPA filters. Permit the glassware and filtration apparatus to dry under the hood upstream of all other operations. Preferably, the hood should be located in a separate room that is supplied with filtered air-conditioned air and maintained under positive pressure with respect to the surrounding areas.

Prior to conducting the test, clean the work surfaces of the laminar flow enclosure with an appropriate solvent. Maintain airflow velocity at 90 ± 20 feet per minute. Prepare the test specimens in the following sequence. Remove outer closures, sealing bands, and

any loose or shedding paper labels. Rinse the exterior of containers with filtered distilled water as described under *System Suitability* for *Determination of Particle Counting Accuracy*. Withdraw the contents of the containers in the normal or customary manner of use or as instructed in the package labeling, except that containers with removable stoppers may be sampled by removing the closure and emptying the contents into a clean container or into the filtration funnel.

The number of samples chosen must be adequate to provide a statistically sound assessment of whether a batch or other large group of units represented by the samples meets or exceeds the limit. For containers of less than 25 mL in volume, test a solution pool of 10 or more units. Single SVI units may be sampled if the individual unit volume is 25 mL or greater. For LVI, single units are tested, but the statistical strength of the estimate of batch suitability obtained must meet the same criteria as a 10-unit pooled sample.

MICROSCOPE PREPARATION

Place the auxiliary illuminator close to the microscope stage, focusing the illuminator to give a concentrated area of illumination on a filter membrane positioned on the microscope stage. Adjust the illuminator height so that the angle of incidence of the

light is 10° to 20° with the horizontal. Using the internal episcopic brightfield illuminator, fully open the field and aperture diaphragms. Center the lamp filament, and focus the microscope on a filter containing particles. Adjust the intensity of reflected illumination until particles are clearly visible and show pronounced shadows. Adjust the intensity of episcopic illumination to the lowest setting, then increase the intensity of episcopic illumination until shadows cast by particles show the least perceptible decrease in constants.

OPERATION OF CIRCULAR DIAMETER GRATICULE

The relative error of the graticule used must initially be measured with an NIST certified stage micrometer. To accomplish this, align the graticule micrometer scale with the stage micrometer so that they are parallel. (Compare the scales using as large a number of graduations on each as possible). Read the number of graticule scale divisions (GSD) compared to stage micrometer divisions (SMD). Calculate the relative error using the formula:

$$(GSD - SMD)/SMD \times 100.$$

A relative error of ± 2% is acceptable. The basic technique of measurement applied using the USP Circular

Diameter Graticule is to mentally transform the image of each particle into a circle and then compare it to the 10- and 25-µm graticule reference circles. The sizing process is carried out without superimposing the particle on the reference circles; particles are not moved from their location with the graticule field of view (the large circle) for comparison to the reference circles. Use the inner diameter of the clear graticule reference circles to size white and transparent particles. Use the outer diameter of the black opaque graticule reference circles to size dark particles.

Rotate the graticule in the right microscope eyepiece so that the linear scale is located at the bottom of the field of view, bringing the graticule into sharp focus by adjusting the right eyepiece diopter ring while viewing an out-of-focus specimen. Focus the microscope on a specimen looking through the right eyepiece only. Then, looking through the left eyepiece, adjust the left eyepiece diopter to bring the specimen into sharp focus.

PREPARATION OF FILTRATION APPARATUS

Wash the filtration funnel, base, and diffuser in a solution of liquid detergent and hot water. Rinse with hot water. Following the hot water rinse, apply a second rinse with 1.2 µm or finer filtered distilled deion-

ized water using a pressurized jet of water over the entire exterior and interior surfaces of the filtration apparatus. Repeat the pressurized rinse procedure using 1.2 µm or finer filtered isopropyl alcohol. Finally, rinse the apparatus with 1.2 µm or finer filtered distilled deionized water using the pressurized rinser, and allow to dry thoroughly prior to use.

Assemble the cleaned filtration apparatus with the diffuser on top of the filtration base, placing the clean membrane filter on top of the diffuser. Remove a membrane filter from its container using ultracleaned blunt forceps. Use a low pressurized stream of 1.2-µm or finer filtered distilled deionized water to thoroughly wash both sides of the filter by starting at the top and sweeping back and forth to the bottom. Place the funnel assembly on top of the filtration base and lock it into place.

Test Procedure

TEST PREPARATION

Liquid Fill (LVI or SVI)Thoroughly mix the units to be tested by inverting 20 times. Clean the outer surface of the solution container thoroughly with pressurized 1.2 µm or finer filtered distilled deionized water. Open the units in a manner consistent with generation of lowest possible numbers of background particles.

For products of less than 25 mL volume, in a cleaned container, open and combine together the contents of 10 containers. Units of greater than 25 mL volume are filtered separately.

Fill the filtration funnel with test solution and apply vacuum. [NOTEDo not let fluid volume in the filtration funnel drop below 100 mL between refills.] After the last addition of solution, begin rinsing the walls of the funnel by directing a low pressure stream of filtered distilled deionized water in a circular pattern along the walls of the funnel and stop rinsing the funnel before the volume falls below 25 mL. Maintain the vacuum until all the liquid in the funnel is gone. Turn the vacuum off, and remove the filtration funnel from the filtration base, removing the filter with blunt forceps to a Petrislide. Secure in place with double stick tape and label with sample identification. Allow the filter to air dry in the clean bench with the cover ajar.

NOTEIf a SVI unit of 25 mL or a solution pool of less than 25 mL total volume is to be tested, add to the funnel a sufficient volume of diluent to give approximately 100 mL total volume. After adding the pooled material, apply vacuum and proceed with filtration as specified above for a liquid solution unit.

Dry Powder VialsReconstitute the material with

an appropriate diluent using the method least likely to introduce extraneous contamination. Add the re-constituted solution to the filter funnel atop a sufficient volume of 1.2 µm filtered water to bring the total volume to be filtered to approximately 100 mL. Pool the desired number of units and proceed as directed under *Test Procedure*.

Solution PoolsFor products packaged in containers that are constructed to hold the drug product and a solvent in separate compartments, mix each unit as directed in the labeling, activating and agitating each unit in order to ensure thorough mixing of the separate components. Analyze the solutions as described under *Liquid Fill* (*LVI or SVI*).

Multiple-dose ContainersFor products labeled as Pharmacy Bulk Package not for direct infusion, proceed as directed under *Liquid Fill* (*LVI or SVI*), performing the test on sample aliquots taken from single units that are equal to the maximum dose given in the labeling. For the calculations below, consider this portion to be the equivalent of the contents of one full container. For products packaged in containers that are constructed to hold the drug product and a solvent in separate compartments, mix each unit as directed in the labeling, activating and agitating each unit so

as to ensure thorough mixing of the separate components.

Enumeration of Particles

The microscopic assay described in this section is flexible in that it can count specimens containing 1 particle per mL as well as those containing significantly higher numbers of particles on a per mL basis. This method may be used where all particles on an analysis membrane surface are counted or where only those particles on some fractional area of a membrane surface are counted.

TOTAL COUNT PROCEDURE

In performance of a total count, the graticule field of view (GFOV) defined by the large circle of the graticule is ignored and the vertical crosshair is used. Scan the entire membrane from right to left in a path that adjoins but does not overlap the first scan path. Repeat this procedure, moving from left to right to left until all particles on the membrane are counted. Record the total number of particles $\geq$ 10 and $\geq$ 25 µm. Calculate the particle count for the unit tested, in particles per mL, taken by the formula:

$$P/V,$$

where P is equal to the total number of particles counted, and V is the volume of the solution in mL.

PARTIAL COUNT PROCEDURE

If a partial count of particles on a membrane is to be performed, the analyst must first ensure that an even distribution of particles is present on the membrane. This is assessed by rapid scanning in order to look for clumps of particles. None should be present. Count the particles ≥ 10 µm in one GFOV at the edge of the filtration area as well as those in the center of the GFOV. The number of particles ≥ 10 µm in the GFOV with the highest total particle count should not be more than twice that of the GFOV with the lowest particle count. A filter failing these criteria is rejected and another prepared if a partial count procedure is used. Alternately, this membrane may be analyzed by the total count method.

The normal number of GFOV counted for a partial count is 20. If a smaller confidence interval about the result is desired, a larger number of fields and particles may be counted. Count all particles that have a circular area diameter ≥ 10 µm and ≥ 25 µm within the GFOV and those that are in contact with the right side of the GFOV circle. Do not count particles outside of the GFOV. Ignore those that touch the left side of the GFOV circle. The dividing line between right and left sides of the GFOV circle is the vertical cross hair. [NOTEMake the best possible judgment

on particle size without changing the microscope magnification or illumination.]

The analyst may increase or decrease the number of fields counted to achieve a desired confidence interval about the count obtained. Calculate the confidence interval about the number of particles per mL ≥ 10 µm and ≥ 25 µm taken by the formula:

$$\sqrt{P},$$

where *P* is the number of particles counted.

In the event that filtration of a solution results in a particle count too high to accurately enumerate in a GFOV, a single quadrant of the GFOV may be counted using a 47-mm filter and a fractional aliquot of the unit. To perform a partial count of this quadrant, start at the right center edge of the filtration area and begin counting adjacent GFOVs. When the left edge of the filtration area is reached, move one GFOV toward the top of the filter and continue counting GFOVs by moving in the opposite direction. Moving from one GFOV to the next can be accomplished by one of two methods. One method is to define a landmark (particle or surface irregularity in the filter) and move over one GFOV in relation to the landmark. A second method is to use the vernier on the microscope stage to move one millimeter between GFOV. To facilitate the lat-

ter, adjust the microscope x and y stage positioning controls to a whole number at the starting position at the center right edge of the filtration area, then each GFOV will be one whole division of movement of the x stage positioning control. If the top of the filtration area is reached before the desired number of GFOV is reached, begin again at the right center edge of the filtration area one GFOV lower than the first time. This time move downward on the membrane when the end of a row of GFOV is reached. Continue as before until the number of GFOV is complete.

If a partial count procedure for the ≥ 10-µm and ≥ 25-µm size ranges is used, calculate the particles per mL taken by the formula:

$$PA/V,$$

where P is equal to the total number of particles counted, A is the filtration area of the membrane in mm2 per graticule fields of view area, and V is the volume of solution filtered in mL.

For a solution pool (SVI units <25 mL in volume) or a single SVI unit, calculate the number of particles taken by the formula:

$$PA/N,$$

where N is the number of unit equivalent volumes filtered.

Interpretation

The injection meets the requirements of the test if the number of particles present does not exceed the values listed in Table 2.

Table 2. Microscopic Method Particle Count.

:	≥10 µm	≥25 µm
Small-volume Injections:		
Powders	2500	250 per container
Liquids	1000	100 per container
Large-volume Injections:	12	2 per mL

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