and materials are disinfected in stages or encapsulated in previously sterilized gowns, gloves, hoods as attempted with personnel for entry. Their level of disinfection and aseptic condition should be equal or better than the environment in which they are used. From the core to the supporting rooms there is a cascading air overpressure for facilities engaged in filling nontoxic (to filling personnel) drug products. In conjunction with the cascading pressure differentials from room to room, there must be sufficient air changes per room to ensure nonviable and viable particulates are controlled to specified levels. The air is supplied through HEPA filters and the air flows, and exchange rates must be justifiable and aligned with regulatory requirements. Air flows from higher graded or classified areas to lower graded areas, that is, class 100, class 10,000, class 100,000 depending on requirements (USFDA guidance classification cited). Rooms themselves must be composed of smooth, nonshedding materials impervious to disinfectants or the exposure to ultraviolet radiation that may be used in the disinfection process.

Using this foot print as a supporting backdrop it is then necessary to superimpose the process flow dynamics to the facility. Drug substance in and drug product out. Materials and clean and disinfected equipment in and "dirty" equipment and wastes out. If the manufacturing process requires support by people necessary personnel must enter and leave. This is to be accomplished without compromising clean with "dirty" and within the confines of the physical plant and established room classifications. It represents the challenge to maintain the aseptic core and core supporting areas as contamination free. This is the complexity of aseptic processing that gives it the high risk status it is known for.

Sanitization and Disinfection

An integral part of any contamination control program is facility cleaning and disinfection practices. The program must be sufficiently robust to maintain the environment in a steady state of microbial control on the basis of criticality of operations and classification designation. The program must be designed to account for any routine variability to the day-to-day function and be flexible enough to respond to the nonroutine perturbations that may challenge the program. Trending and interpretation of both viable and nonviable control data will provide the evidence to make "mid-course" corrections in a well designed and implemented program. Fortunately there are guides available and experience to help determine anticipated frequency and concentrations of cleaning and disinfecting solutions to attain validation goals and maintain control. Adjustments can be made on the basis of data. Selection of agents should be made on the basis of usual, "normal," flora and control of biotypes. Application of disinfectants is typically done on a manual basis with regiments and schemes available from industry benchmarks and vendor information.

Much discussion has been devoted to the topic of rotation of disinfectants and the "need" to control the adaptation of microbes to the mode of action of a disinfectant. It is now generally understood and agreed that the ability of microbes to adapt to the mode of action of a disinfectant and gain "resistance" is negligible or nonexistent. However, it is acknowledged that by not rotating disinfectants there can be a "selection" process for those microbes not susceptible to the mode of action of the chemical of use. It has been shown and is logical that naturally resistant microbes can persist and potentially increase in number by not rotating disinfectants. This has been demonstrated with in vivo data of endospore formers in the presence of quaternary ammonium compounds versus sporicidal agents to which they are susceptible.

Environmental Monitoring System

Evaluating the quality of air and surfaces in a clean room environment should start with a well-defined and written program. PDA TR #13 revised (2001) (12) as a starting point will provide a variety of information to help develop a program suitable to your facility and aligned with industry practices. Other reference documents such as ISO 14644, clean rooms, and associated controlled environments should be considered (13). Methods employed should be qualified/validated prior to implementation. The number of sampling sites will vary depending on the design of the area and the clean room technology employed (conventional filling line, RABS, or isolator). An aseptic core utilizing a conventional filling line would

present the greatest control risk due to much more operator interaction and intervention. With employment of more restrictive technologies such as RABS or isolators, human contact would be reduced or virtually eliminated and hence the number of sampling sites could be reduced accordingly. The program should address all shifts and include all exposed surfaces of the room and equipment and include critical surfaces that come in contact with the product, containers, and stoppers. Sample timing, frequency, and location should be carefully selected on the basis of their relationship to the operations performed. Recent application of risk-based approaches to site selection and decision making has gained acceptance.

It is useful to utilize current approaches emphasizing good science and risk-based approaches to contamination control. Application of Quality Risk Management (QRM) approaches as delimited in documents such as ICH Q9, and tools that support such approaches can be utilized effectively for such purposes. Identifying contamination/risk points in your process stream and applying a QRM mindset will document the thought and support the process for establishing your program. Emphasis should be placed on contamination caused by operator interaction/intervention and at intervals in the process where there is the intersection of inputs and outputs into and from the core area. These transition points are most vulnerable to contamination introduction. These assessment points will be indicative of your operation, and there can be variability from line to line or between facilities. Each will exhibit its own "fingerprint" for usual microbial flora, their numbers, and location of recoveries, and is influenced by disinfection program, facility layout, work flow, that is, the total control program. This fingerprint is developed from trended data. Although some variability of such data is normal the level of control should be sufficiently robust and sufficiently detailed to recognize trends indicating a perturbation in control and potential risk. Implementation of a total control program provides sufficient control to maintain operations within guidance or regulatory levels.

Air Monitoring, Nonviable and Viable

Any comprehensive environmental control program should include both nonviable and viable monitoring. There has always been some discussion about the potential interrelatedness of the data generated from each activity; the position microorganisms need carriers such as nonviable particles for dispersal has won both skeptics and advocates. On that topic the evidence seems to support the premise that you must have particles for microbial transfer but not all particles necessarily carry microbes. This premise has also been reflected in the argument supporting both 0.5 and 5.0 μm particle monitoring. I do not think any one would refute the basic premise of microbes "piggy backing" on particles but to say HEPA (high efficiency particulate filters) filtered air is a significant source of microbes is questionable. In any case it is generally recognized that some level of interrelatedness is apparent and warrants the pursuit of control with both approaches, nonviable and viable contributing to the total program. A great injustice would be done by looking at the data so prescriptively that the value of the information is lost in the argument over the interpretation of the details. Examining the inside out approach to environmental control described earlier, core to lesser controlled areas, it is evident that monitoring data generated in areas of lesser control and consequently "closer" to an uncontrolled state are more difficult to interpret than data generated in more highly controlled areas, e.g., class 100 compared to class 100,000 since the frequency of testing tails off sharply as we move out from the class 100 area.

Data Interpretation

Environmental monitoring is for the most part not an exact science but it represents our best attempt, given the tools currently available to us, to help characterize an environmental control program. Individual data represents a static point at which the data is collected, a snapshot in time is the terminology sometimes used. The totality of that data, all the data points collected, typifies the level of control during the operation on any given day at any given time. No one data point absolutely defines the conditions or level of control of a fill or of the total program. If the total control program is designed and implemented appropriately no single data point would indicate a process breakdown unless there was a catastrophic failure. That scenario would be the best case situation verifying the program as implemented is working as intended.

Single events should typify an isolated incidence that could be assessed through investigation and risk assessment to determine impact. Since the level of control often varies on the basis of the type of technologies employed and operator interaction with that technology, some variability in data can be expected. Each data point and the collective array of data should be evaluated against the data history for the line, area, or process to determine consistency in control levels. Alert and action levels should be established and a mechanism to identify deviations and formulate responses should be clearly elaborated. Responses should be science-based and appropriate to the potential risk presented by the deviation. Responses should be holistic in approach as well as mitigating the immediate risk posed by any event keeping in mind the lag time between the event and the results.

Aseptic processing is based on the separation of the filling environment from potential contamination and any inputs to the process stream including operators. With the technologies available, and considering the conglomeration of life on this planet, this is essentially done in a somewhat arduous and cascading manner. The least areas of control are furthest from but connect to the greatest areas of control. The areas of greatest control are the class 100/class 10,000 core area, using USFDA terminology, which are the cleanest and the most free of contamination and furthest from the natural uncontrolled world. Through that connection and by devising increasing levels of environmental control outside to inside, from natural uncontrolled areas to highly controlled core area, maintenance of a class 100 area can be accomplished. Much attention is given to the control of that class 100 area and understandably so, but it is often overlooked or underestimated that inputs in your process stream including operators must traverse the lesser controlled areas to perform the end functions of filling and stoppering of the product. What happens upstream will decidedly have an impact on operations downstream. By sequentially cascading areas, from nature outside the physical plant to unclassified area inside the plant to various levels of classification, we achieve what is regarded as a suitable level of control of the area to accomplish aseptic processing. It is not considered the best of situations by regulators for product manufacture, but when the nature of the drug product does not permit sterilization by other accepted technologies, aseptic processing is permitted for manufacture of sterile products.

When something happens that impacts our control it should be detected. If it is not detected it may indicate we are not "measuring/monitoring" the appropriate indicators of change for the area. Since major control parameters can be more readily measured, it is usually the subtle events, those not directly measured or detectable, that impact control. That underscores the importance of looking for trends. Often our concentrated efforts are at the immediate point of focus and do not consider the control of areas and operations cascading from areas of lesser control, and as a result we struggle with appropriate corrective actions. Having said that, it is not reasonable that we monitor less critically controlled areas with the same rigor that we monitor class 100 areas but in an effort to get meaningful data from less controlled areas we must have greater assurance that what we do monitor from those areas adds value to the program. In many instances decisions of when and where and even possibly who we monitor in lesser controlled areas are not scientifically sound and not as well conceived as what we do for class 100 areas. Of course there is more prescriptive guidance on what to do in the class 100 area and frankly in some instances we have adopted such guidance to lesser controlled areas without thinking through our actions. Here is where the QRM process and a risk-based assessment can greatly enhance the value of our monitoring activity and make our control programs more robust. In numerous instances, using a QRM approach to improve control in our lesser controlled areas has decreased costs through reduced monitoring and manpower expenditures and at the same time increased its value because of acquisition of more meaningful data.

If data generated has greater capability to indicate perturbations in microbial control we should be able to respond in a more deliberate and effective manner. To do so this requires a more in depth review of data and a more frequent review of data. Not only is a response required at the point of deviation but one should also look at the potential impact upstream and downstream of that event. This expanded look and response could be proactive as well as reactive even if further contamination is not detected. We tend to be too focused in our reviews and responses on specific events and as a result subtle irregularities were not seen until they

became events and were widespread and problematic. This has been exacerbated due to the delay in getting data from monitoring to detect them they must grow. Reaction time is the cornerstone of control.

With our current capabilities even for the most controlled environments it is broadly recognized that interpretation of data in the vast amount of cases can not be directly extrapolated to product risk. To deliver value in most instances the entire control picture must be formatted and analyzed to get an understanding of what if anything is going on microbiologically, knowledge versus information. This necessitates looking at data in other ways then the daily format associated with the collection of information. Trends, departure from routine or expected data, can best be recognized by grouping information into related categories and looking for changes. Multiple approaches to grouping information can be used to assist with change identification. Groupings focused by room, by classified area, and by microbial characteristics are but a few. Microbial characteristics such as Gram reaction, Genus and species identification where and when appropriate, and numbers isolated in CFUs are common approaches. The transition between classified areas, airlocks, and pass-throughs are excellent areas where potential issues can be identified as they are developing. Changes in any one of these parameters may have significance in the overall microbiological maintenance/control of the facility. Frequency of review is paramount to identification of trends, events, or perturbations in the control program. Generally, the more data the better the capability of analysis but more is only useful unless it is relevant and if it is analyzed. Data collection for data's sake is not productive. If you have information then you must do something with it and that it adds value to the program or it is not worth collecting. If it does not add value to your control program then do not pursue it.

Where or when does environmental monitoring not add value? There is a movement of late to monitor areas remote from the classified areas. The data generated may be taken at long/infrequent intervals. Such data adds little knowledge to the maintenance of the control process and is likely to expend significant manpower and cost to generate the data. A well-devised and implemented facility cleaning and control program as described earlier will contribute more to maintenance of facility control than environmental monitoring. If microbial monitoring is to be done in support of unclassified area control, the application of microbial acceptance criteria to cleaning and sanitization validation would be appropriate. Routine monitoring at long intervals is not scientifically sound and adds little value. The best approach is to incorporate a risk-based approach to decision making when devising a total program. Use of tools like HACCP has been shown to be effective in such applications.

There are multiple regulatory or guidance recommendations for periodicity of data collection. Recommended frequency of data collection is generally higher in more controlled areas and less in lesser controlled areas. However, since facility equipment and personnel control is basically from the outside in, class 100,000 → 10,000 → 100 or grade D > C > B > A, whatever scheme you choose to follow, it might be advisable to monitor or review data with greater frequency in the outer areas to give a more dynamic picture and information that can be the key to response and subsequent control. What appears outside by design generally works its way to the inside. Actually frequencies of data collection and review should be flexible based on the operations they are intended to control and the data recovered for analysis. To maintain control in a cascading system, it is most effective to put an emphasis on the upstream process to mitigate issues before they get to your critical areas. This is a position that is now being stressed in guidance information but is not always heeded by QA/QC units. Regulatory requirements or guidance recommendations should be regarded as minimums and adjusted to fit your circumstances and total control program.

How to Handle Excursions

Like any deviation to requirements an investigation into the cause is expected. The extent of the investigation should be commensurate with the event and its proximity to the core A/B classified area or potential impact on the process. Investigations should have some consistency in approach or scope again based on criticality. Identified trends, alert level excursions, and action level excursions can be treated differently but must be defined by procedure. A rationale based on science and risk should support the position. Investigation should be systematic in

approach and of sufficient scope to determine potential impact both upstream and downstream of the event and between recently manufactured batches. All investigations having the potential for batch implication should be closed and reviewed by the quality unit prior to batch release.

Process Simulation—Media Fills

By definition media fills are designed to mimic the manufacturing process. They also represent an opportunity to collect additional data and knowledge about aseptic control mechanisms in your process. During media fills we are qualifying operators and stressing our processing environment by running our processes to their defined limits both upper and lower, both mechanically and by simulating in a condensed format the extremes of anticipated operating conditions. By doing added environmental and personnel monitoring during these exercises we can determine when and where risks are elevated due to the added interactions of personnel with the equipment. Not only do these activities test the robustness of your aseptic process, but the information obtained can give additional insight into where and when microbial risk arises with activities. From such data modification of location or timing of EM can be enhanced. Recall, however, that media fills are to represent the normal activities during the process and are not meant to justify practices that pose an unnecessary contamination risk. During routine aseptic manufacturing conditions you would want to do the minimum amount of monitoring since the activity in and of itself introduces a level of risk. Media fills give the latitude to explore that arena and apply leanings.

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4 | Water systems for parenteral facilities

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INTRODUCTION

The most challenging pharmaceutical water applications are typically associated with parenteral products since, by their nature, parenterals are uniquely able to access bodily fluids and tissues. In this aqueous environment, water is uniquely suited to serve in a multitude of roles associated with drug development, testing, manufacture, and delivery.

Because of their unparalleled access to critical areas of the body, and compounded by their irretrievable nature, parenterals must meet extremely stringent requirements both in the United States and around the globe. Injectables necessitate the use of water that is chemically and microbiologically pure, to exacting standards, both from a practical perspective and based on regulatory dictates to avoid patient risk and to ensure product and treatment efficacy. As a product, excipient, cleaning agent, solvent, etc., water is used in significant quantities and, in many cases, it is the single largest volumetric commodity associated with any finished product.

This chapter will discuss the uniqueness of parenteral water applications including their current regulatory requirements. Discussion will focus on injectable risks, compendial limits for chemical purity, viable and nonviable microbial contamination, and added substances. Approved water treatment methods vary significantly throughout the world in spite of harmonization efforts and pose a significant hurdle for global firms wishing to reduce costs, consolidate manufacture, and standardize operations. As part of the discussion associated with approved methods of producing parenteral waters, common equipment types, basic system designs, operational challenges, and delivery/utilization issues will be reviewed. Of paramount concern is the ability to design, install, operate, and maintain a system that will consistently produce suitable quality water. Sanitization, testing, and monitoring are a few of the other key items that will also be addressed.

WATER GRADES

There is a considerable number of water grades used for pharmaceutical applications with varying regulatory requirements. The most significant of these are tabularized with their primary criteria in Table 1. These various grades are best characterized by their use, falling primarily into two groups, within which both bulk and packaged waters are defined. It should be noted that these two primary groups are identified by their role relative to parenteral products, such that one is specifically designated *for* parenteral use while the other is designated *not for* parenteral use. Hence, it will be most practical to begin the discussion of water types and their application by reviewing the two primary types of bulk water, followed by discussion of each individual packaged grade including the requirements that make each unique and specialized.

BULK PHARMACEUTICAL WATERS

Bulk waters are those waters produced by pharmaceutical manufacturers for use in or during production of their products and usually within their facility, while packaged waters are waters typically produced for incorporation into limited sized containers, most often one liter or less, and sold as a finished product for use in a multitude of applications ranging from extemporaneous compounding to laboratory testing. Packaged waters are most often sterilized to ensure that any residual microbial contamination does not multiply out of control, resulting in a compromised product or injured patient and owing to a general avoidance of preservatives.

Sterility is not required for bulk water, including Water for Injection (WFI); however, responsibility is placed on the drug manufacturer to ensure the safety and efficacy of their products. This reliance is monitored by regulatory bodies tasked with protection of the public health. The lack of a sterility requirement for bulk water is not based on a lack of concern but instead on the recognition that WFI in bulk form will often require additional processing, and

Table 1 Water Grades with Monograph Designation of Applicable Standard (Water for Hemodialysis Has Been Omitted)

	Water for njection	Water Highly Purified[a]	Bacteriostatic Water for njection[b]	Sterile water for nhalation[b]	Sterile water for njection[b]	Sterile water for rrigation[b]	Purified Water	Sterile Purified Water
Bacterial endotoxins <85>	<0 25 EU/mL	<0 25 U/mL	<0 5 EU/mL	<0 5 EU/mL	<0 25 EU/mL	≤0 25 EU/mL	NA	NA
Total organic carbon <643>	Meets the requirements	Meets 2 2 44	NA	NA	NA	NA	Meets the requirements	NA
Water conductivity <645>	Meets the requirements	Meets the requirements	NA	Meets the requirements	NA	Meets the requirements	Meets the requirements	Meets the requirements
Packaging and storage	NA	NA	Specific requirements per monograph	Specific requirements per monograph	Specific requirements per monograph	Specific requirements per monograph	NA	Specific requirements per monograph
Labeling	NA	NA	Specific requirements per monograph	Specific requirements per monograph	Specific requirements per monograph	Specific requirements per monograph	NA	Specific requirements per monograph
Antimicrobial agents <51> & <341>	NA	NA	Meets effectiveness and Content	NA	NA	NA	NA	NA
Particulate matter <788>	NA	Clear and colorless	Meets the requirements	NA	Meets the requirements	NA	NA	NA
pH <791>	NA	NA	Specific requirements per monograph	NA	Specific requirements per monograph	NA	NA	NA
Sterility <71>	NA	NA	Meets the requirements	Meets the requirements	Meets the requirements	Meets the requirements	NA	Meets the requirements
Other requirements	NA	Specific Requirements per EP	Specific requirements per monograph	Specific requirements per monograph	Specific requirements per monograph	Specific requirements per monograph	NA	Specific Requirements per monograph
Microbial guidance <1231>	≤10 CFU/ 100 mL	≤10 CFU/ 100 mL	Meets the requirements	Meets the requirements	Meets the requirements	Meets the requirements	≤100 CFU/mL	Meets the requirements

[a]Column is only applicable to the European Pharmacopoeia 6 3 (USP designations are not applicable)
[b]Products produced from bulk Water for njection (WF)

sterility on such a large scale is difficult, if not impossible, to achieve and/or prove. This situation may be compounded by testing, often in uncontrolled environments. Hence, it is frequently more practical to control final sterility using one or more alternative methods such as portion sized terminal sterilization or sterilization via filtration, all of which can be assured at a higher level of reliability than the bulk counterpart.

Sterilization reliability is primarily based on statistical methods as absolute testing for sterility is effectively impossible with the technology currently available. As a result, sterility can be achieved but cannot be practically measured on a direct quantitative basis without compromising the sterility itself or destruction of the product. This is complicated additionally by the nature of microorganisms as living entities and their lifecycle tendencies including their reproductive processes, their resilience, their potentially unique and individual response to stressed conditions, and their ability to colonize, just to name a few of the traits that improve their survivability and make sterilization quite challenging.

Discussion of microorganisms and biofilm will occur further on in this chapter to provide a basic understanding of those challenges associated with biologic control of water used in, and for, pharmaceutical product manufacture, as well as the testing required to ensure the suitability of water intended for use in parenteral products. Traditional cultivative pour-plate methods that are typically employed result in lengthy delays before data is available, causing slower than optimal reaction to failures or anomalies and implementation of less than efficient quarantine practices to ensure safety and efficacy prior to product release. System configuration can affect the ability to obtain valid cultivative data compounding an already difficult situation.

Microbiologists familiar with pharmaceutical water system design, operation, sanitization, and, most importantly, the flora likely to exist in a particular system should be consulted when methods and practices are established for a new system and also throughout the life of the system, as changes can occur over time and as the system, and its resident biofilm, change.

Ultimately, controlling biofilm formation and growth are the most critical aspects of water system microbiologic performance, and certain designs are better than others in this regard. Only through knowledge and understanding can system designers, operators, and quality personnel assure that a system is suitable and functional for the intended purpose. In spite of what many may think, water isn't "just water" as it comes from the faucet, water is a critical utility without which pharmaceutical manufacturing might quickly grind to a halt, resulting in significant financial impact. Water may be designated as a critical raw material essentially manufactured on site and often used without the review and approval of Quality Control (QC), unlike that required for virtually all other raw materials. It is extremely important that adequate water is available for manufacturing but it is even more important that the water is of the required quality.

As mentioned, bulk water is primarily used during product manufacture; however, it is important not to overlook the fact that both WFI and Purified grades of water are also available in packaged form for extemporaneous and other uses. As such, there must be recognition and understanding of the difficulties associated with packaging, storage, and use beyond the confines of a controlled manufacturing environment.

Using the current US Pharmacopeia Revision 32 as our compendial reference, the two primary types of bulk pharmaceutical water are designated; Purified Water, USP and Water for Injection, USP. Each of these water grades is defined monographically and must meet specifications for quality and purity; however, certain critical aspects are left somewhat to the discretion of the drug product manufacturer, based on guidance provided in informational sections of the compendium. Often this guidance, which includes recommended standards, is enforced by regulatory agencies along with those mandatory specifications listed in the monographs themselves. Such is the case for microbial action levels (and alert levels although no numeric value is delineated), included in information section <1231> of the USP, as FDA enforces these at least as strictly as the actual requirements presented in the monographs.

It is worthy of note that the US Pharmacopeia is prepared and published by an independent not-for-profit corporation that is not affiliated with the United States government, yet the resulting specifications, called monographs, are recognized as law, based on action taken by the US Congress beginning with the Food, Drug & Cosmetic Act of 1938. The US Food

and Drug Administration (FDA), part of the Department of Health and Human Services, is tasked with enforcement of these specifications. However, the FDA does not have authority to make changes to these regulations or to promulgate new ones.

Purified Water, USP is defined within the US Pharmacopeia as "...water obtained by a suitable process." and "...prepared from water complying with the U.S. Environmental Protection Agency National Primary Drinking Water Regulations (EPA-NPDWR) or with the drinking water regulations of the European Union, Japan or with the World Health Organization's Guidelines for Drinking Water Quality (1)." This water must contain no added substances and must meet limits for total organic carbon per USP <643> and conductivity per USP <645>. As well, Purified Water must be protected from microbial proliferation and monitored using suitable action and alert levels to ensure control and use of only suitable quality water. Purified Water *is not* to be used in preparations intended for parenteral administration but rather, it is intended for use as an ingredient and in tests and assays as appropriate. The USP monograph for Purified Water does not include limits for endotoxin; however, the European Pharmacopoeia (EP) has recently added a grade of water designated as "Water, Highly Purified (2)" (Aqua valde purificata) that does include an endotoxin specification limit; however this new grade is not intended to replace WFI, but rather to create a grade between Purified and WFI. In addition, a number of companies, based on their product requirements, have implemented internal specifications that effectively add endotoxin limits to Purified Water specifications. Some of these Purified Water applications, such as for ophthalmic solutions (used during surgery for cataract removal or Lasik) and inhaled products, may require endotoxin control, based on the nature of the use.

WFI, USP is defined within the US Pharmacopeia as "...water purified by distillation or a purification process that is equivalent or superior to distillation in the removal of chemicals and microorganisms (3)." and, "...prepared from water complying with the U.S. Environmental Protection Agency National Primary Drinking Water Regulations or with the drinking water regulations of the European Union, Japan or with the World Health Organization's Guidelines for Drinking Water Quality (4)." This water can contain no added substances and must meet limits for total organic carbon per USP <643>, conductivity per USP <645>, and Bacterial endotoxin per USP <85>. WFI must be protected from microbial proliferation and monitored using suitable action and alert levels to ensure control and use of only suitable quality water. WFI is intended for use in the preparation of parenteral solutions. The chemical purity requirements (both conductivity and total organic carbon) for WFI are the same as those for Purified Water; however, WFI must meet the added requirement for bacterial endotoxin. In addition, the recommended microbial action and alert levels for WFI are 1000-fold more stringent than for Purified Water.

Table 2 summarizes the expectations both monographic (in green) and enforced guidance (in blue) for the bulk grades of pharmaceutical water per the current USP.

Note: USP <645> for conductivity is a multistage testing methodology that allows for compliance under three possible scenarios with the most preferred being stage 1; associated with on-line testing. Off-line testing can also be accomplished using stage 2 and 3 methods; however there is greater chance for sample error using these options. Off-line tests are designed to account for the effects of carbon dioxide and its resultant impact on water quality. Further details can be obtained by referring to the USP or, if appropriate, the applicable regulatory document from another locale.

Endotoxin is, more specifically, a component of the cell wall of gram negative bacteria and properly referred to as a lipopolysaccharide (LPS). These compounds are toxic, eliciting a fever when injected into a patient's tissue or bloodstream, hence the term pyrogen. Patient response can range from a rise in body temperature to a state of shock and even death. "The

Table 2 Summary of Requirements for Bulk Pharmaceutical Waters

	Conductivity	Total organic carbon	Endotoxin	Microbial (NMT)
Purified Water	Per <645>	Per <643> (≤500 ppb)	NA	100 CFU/mL
Water for Injection	Per <645>	Per <643> (≤500 ppb)	< 0.25 EU/mL	10 CFU/100 mL

term "endotoxin" is usually interchangeable with the term "pyrogen," although not all pyrogens are endotoxins, and pyrogen testing alone cannot be used entirely for detection and characterization of microbial endotoxins (5)." Hence, endotoxin must be controlled to the lowest levels possible or removed entirely from any water used in parenteral preparation to ensure patient health and safety. There are two primary test methods suitable for detecting these materials; the USP Pyrogen Test <151>, which is based on a rise in body temperature in rabbits, and the USP Bacterial Endotoxin Test <85>, also known as the *Limulus Amoebocyte Lysate* (LAL) test. LAL options include gel-clotting, turbidimetric, and chromagenic versions all of which rely on the reactivity of horseshoe crab blood cells. All of these test protocols must be performed off-line in a laboratory and require considerable time to produce results, making biologic control in a water system all the more critical.

In summary, Purified Water that complies with the USP can be manufactured using any suitable process and must meet the summary requirements listed above. On the other hand, WFI can be produced by distillation or alternatively, in the United States, the producer must be able to prove, via scientific methods and testing, that an alternate method is equal to, or better than, distillation when employed in a well designed and operated system. Coupled with the requirement by the EP to produce WFI only by distillation, these confines have dissuaded most from the challenge, rendering almost all WFI, produced for use in the United States, distilled, as well as, all WFI regulated by the EP. Notwithstanding, WFI must meet all of the summary requirements listed above for chemical and biologic purity as well as the inferred microbiologic levels enforced by FDA.

PACKAGED PHARMACEUTICAL WATERS

Packaged grades of water, including Bacteriostatic WFI, Sterile Water for Inhalation, Sterile WFI, Sterile Water for Irrigation, and Sterile Purified Water, are all sterile packaged waters typically produced from their bulk counterpart with further processing to meet the requisite monographic requirements and then packaged for subsequent use.

Packaged water can be contained in glass or plastic containers. The container configuration can be rigid as might be the case for vials, syringes, or bottles, or can be flexible as in the case of intravenous injection (IV) bags. Packaged water can be configured for single dose or for multiple dose applications provided suitable preservatives and labeling are employed. Container size, as mentioned, may be limited as is the case for Bacteriostatic WFI (30 mL max.) and Sterile WFI (1 L max.). Other packaged waters such as Sterile Water for Inhalation, Sterile Water for Irrigation, and Sterile Purified Water may be available in larger than 1 L containers based on their use (i.e., Sterile Water for Inhalation used for humidification or Sterile Water for Irrigation used in large volumes during surgery and often designed for rapid emptying). In spite of its sterile condition, Sterile Purified Water is not suitable for preparations intended for parenteral administration.

Glass packages are typically more inert and are traditionally considered to be more pharmaceutically elegant; however it must be understood that only certain grades of glass are suitable for parenterals. Like water, glass is also often designated on the basis of its relation to parenterals such that borosilicate glass is typically most preferred and Type III soda-lime glass is typically identified with the label NP, meaning "not for parenterals." Notwithstanding its benefits and image, glass is often more susceptible to breakage, is usually more costly and weighs considerably more than its plastic counterpart resulting in added cost for shipping, handling and losses from breakage. Plastic, on the other hand, although lighter and less expensive to produce, typically requires higher levels of testing to ensure the product will not be contaminated by, or absorbed by, the package material. Concerns relative to plastics often dictate extended testing for leachables and extractables and to verify product integrity.

There are two methods of producing a sterile packaged product. These methods vary significantly based on their primary techniques. The more highly preferred of the two is terminal sterilization because the product is first sealed in its container and then the entire unit (product and container) is sterilized. This is preferred since completion of the sterilization process produces a higher reliability of final unit integrity. Alternatively, products can be manufactured aseptically whereby a sterile product (e.g., water) and sterile components (e.g., container) are brought together in a controlled environment such as a Class 100 clean space for

final filling and sealing. Both of these methods are effective when properly implemented recognizing the product, container, and any other limiting variables that might be applicable; however, aseptic filling of water products is typically only utilized when terminal sterilization is impractical.

For terminal sterilization, there are five methods listed in USP section <1211>, including steam sterilization, dry-heat sterilization, gas sterilization, sterilization by ionizing radiation, and sterilization by filtration. Although this chapter is not intended to address each of these in detail, the suitability of sterilization method relative to water and its package becomes readily apparent. Dry heat sterilization that results in vaporization of the water or destruction of the container would obviously be inappropriate. As well, gas sterilization relies on permeation through the package with diffusion of the gas into the product. Hence, it is not suitable for all applications, possibly due to residual sterilant in the product or where the container would not allow this to occur, such as with glass vials or bottles or with liquid products such as water.

Alternatively, aseptic processing allows the water to be sterilized by a suitable process (i.e., filtration) and the container, be it glass or plastic, to also be appropriately sterilized before entering the classified space (ISO Class 5, EU Grade A, SI (Metric) Grade M3.5, or previously applicable FS-209E Class 100). The difficulty of aseptic processing remains in the necessity to design and maintain the conditions adequately to ensure a sterile environment.

It is important to note that absolute sterility cannot be tested without complete destruction of the product. Hence, statistical methods are required for determination of sterility and sterilization reliability. The subject of sterility and sterility testing is covered in the USP in detail as is also the case with other regulatory volumes throughout the world. Sterility will not be covered in extensive detail in this chapter.

PHARMACEUTICAL WATER SYSTEM DESIGN

Water used for parenterals (WFI) is produced by distillation in most cases, as previously noted. Whether used in bulk or further processed to create packaged versions, there are basic requirements for the systems that produce this water to ensure that it is of the proper quality and that it can be delivered to suitable locations for subsequent processing as part of a finished product, as a packaged water, for cleaning, for laboratory testing applications, or for other suitable purpose.

Regulatory requirements demand that water complying with EPA NPDWR standards serve as feed water for WFI applications. This requirement is typically considered to be a minimum such that often pharmaceutical manufacturers will include additional treatment steps or will use even higher quality feed water (including Purified Water) to ensure the highest chemical, microbial, and mechanical reliability for the systems that produce WFI. Hence, there are a number of system configurations that are able to reliably produce WFI, a few of which will be discussed in detail, further on in this chapter.

Design of a water treatment system typically requires an analysis of the feed water that will be employed. Although EPA NPDWR regulations are prescriptive, it is important to recognize that virtually no two water supplies are identical in the amount and types of chemical contaminants present. Water supplied from a surface source in the southeast may have significantly higher levels of organic contaminants and suspended solids than water from a deep well in the northwest. Alternatively, deep wells may carry higher levels of dissolved minerals than their shallow or surface counterparts elsewhere. All of these variations still fall within the acceptable confines of potable water meeting the standards for EPA NPDWR and are suitable as feed water.

As a result, the task of the water system designer is far more complex than simply selecting a single treatment regime based solely on flow rate or locale. Analysis of the water contaminants present in the feed stream will dictate the type of pretreatment required and may include components such as filters, softeners, reverse osmosis (RO) membranes, and even chemical injection units for applications ranging from the introduction of sanitants, such as chlorine, to injection of flocculants to improve the effectiveness of other unit operations in their contaminant removal.

Each of the pretreatment steps is typically employed to improve the functionality of subsequent downstream components with distillation typically as the ultimate final unit

Table 3 Contaminant Tests Commonly Applied to Evaluate Feed Water Supplies

1. Dissolved inorganics (cation and anion)
2. Resistivity/conductivity
3. Total dissolved solids
4. Silica and iron (reactive and nonreactive)
5. Barium
6. Strontium
7. Hardness
8. Alkalinity
9. Chlorine and chloramine
10. Total organic carbon
11. PH
12. Temperature
13. Silt density index
14. Particle counts
15. Bacteria level
16. Pyrogens
17. Dissolved gases

operation. For example, a coarse filter may be included prior to an RO membrane. Although the RO is capable of removing coarse suspended contaminants, it is far more cost effective to include a prefilter than to clean or replace the RO membrane on a frequent basis. It is these issues, relating to reliability and cost effectiveness, that often drives the component and unit operation selection process in a water system design.

Discussion of the details relating to feed water analysis is beyond the scope of this volume; however Table 3 provides a list of many of the more common contaminants evaluated during system design and upon which a treatment regimen is based. Not all of these may exist in every Feed stock but if present at, or above, certain threshold levels, these constituents can have a significant impact (usually negative) on the operation of a system inadequately designed for their removal or mitigation. That impact can range from unsuitable water being produced to a need for frequent service or cleaning that results in system outage. In either case product manufacturing can be severely restricted as a result.

On the basis of analysis of the feed water and with consideration given to the experiences and preferences of those at the manufacturing site, certain initial decisions will set the basis for the overall system design. For example, if the manufacturing site is located in the United States but the firm plans to produce products for distribution in the European Union, it is very likely that a single system, based on distillation, will be selected for both. Also, if the feed water contains chlorine (or chloramines) the system pretreatment must address its removal using appropriate methods. Assuming the use of cost prohibitive exotic alloys is inappropriate, most likely either granular activated carbon (GAC) or chemical reaction, possibly using a variant of sodium sulfite, will be most suitable. Carbon is excellent for chlorine removal and is also reliable for removing dissolved organics; however carbon is also notoriously difficult to control microbiologically because of the large surface area, resident nutrients, and sanitization difficulty. Alternatively, sodium sulfite can be successfully applied recognizing it is not a panacea and its use includes both advantages and disadvantages when compared to activated carbon. Therefore, the designer must evaluate all appropriate options, choosing the most suitable selection for a specific application. As well, other factors may play a significant role in the decision. For example, high organic contaminant loading in the feed water might indicate a preference for the use of GAC versus injection unless other factors are controlling. Hence, the use of a specific treatment technology may be more or less desirable based on individual system circumstances rather than being based on an ideal theoretical design model. Above all it must be remembered that there are almost always multiple approaches that achieve the same end result. For example, a GAC filter may be used when high organic levels are present; however, sodium sulfite combined with an organic scavenger resin may prove equally effective and more desirable under certain circumstances.

These types of process decisions are present at every step of system design; yet in many cases a suitable basic composite design can still be considered provided the selection is supported by technical review and confirmation for applicability. The following sample system designs are included below to depict common configurations that can be suitable for production of WFI. These are not meant to be universally applicable models but rather to offer examples of frequently used frameworks on which many successful designs have been based. The schematics have been simplified for ease of review and do not contain the level of detail necessary to configure a system properly. These options provide typical design scenarios ranging from very basic to relatively complex acknowledging a plethora of options in between and beyond.

Implementation of these designs can be additionally complicated by site requirements for automation, including data acquisition and trending. It should not be inferred that simpler designs are less costly, as again this may be affected by any number of variables associated with capital acquisition, installation, and system operation. Additional reference material is available in volumes written by authors such as Meltzer et al. (6), as well as in guides such as those produced by the International Society for Pharmaceutical Engineering (7) (ISPE).

Additionally, the cost to validate any pharmaceutical water system is significant, accounting for a large portion of the budget necessary for project completion. Validation, which more recently has been referred to as commissioning and qualification (C&Q), is the verification that a system can and does produce water of the proper quality and may include activities such as design qualification (DQ), installation qualification (IQ), operational qualification (OQ), and performance qualification (PQ), using tools such as commissioning, factory acceptance tests (FATs), and site acceptance tests (SATs).

The design depicted in Figure 1 would be best described as one of the more complex conceptual arrangements, as it provides feed water to the still that will usually meet Purified Water quality requirements. Hence, there is greater complexity with the inclusion of additional unit operations prior to distillation. This format uses RO to prepurify the water fed to the still, reducing the load on the distiller. This design is common when the facility requires both grades of water, based on feed water characteristics, or when dictated by economic or other factors. The common result of this design is a more robust pretreatment system with less challenging demands placed on the still. This robustness is only available based on added cost associated with the purchase and proper operation of additional equipment, such as the RO. Considering the high quality of the feed water presented to the still, in most cases, any type of distiller would function although it would be more likely for traditional distillation technology (i.e., single effect or multiple effect types) to be implemented based on the possible added cost

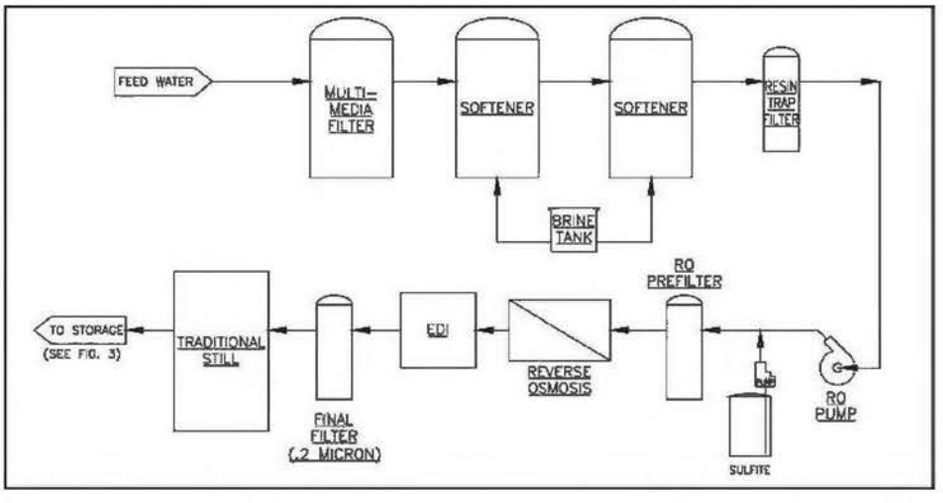

Figure 1 Schematic of a typical traditional still application with Purified Water feed.

associated with a vapor compression still. However, it should be noted that traditional distillation technology typically requires higher quality feed than vapor compression (VC) style stills, to ensure reliable operation, on the basis of operating temperature. For the design shown in Figure 1, the cost of a more complex pretreatment system with its associated higher operating cost partially offsets the lower purchase and operating costs associated with a traditional still design.

In summary, a multimedia depth filter removes coarse contaminants (typically an industrial version of the sand filter used for a backyard swimming pool) and supplies water to dual series softeners that in turn ensure hardness, which would foul the RO, and ammonia, which would pass through the RO are eliminated. The duplexing of this unit operation allows regeneration of one column without the risk of passing such contaminants to the RO. A common brine system, used for regeneration of the ionic softener resin, alternates between columns. A cartridge prefilter protects the RO feed pump from resin and fines while a pre-RO filter protects the membranes and provides additional filtration. The RO feed pump generates the pressure necessary for RO membrane operation with pressures typically in the range of 200 to 600 psig. Sodium sulfite is injected prior to the RO, to remove residual chlorine from the municipal source, which was allowed to remain since it assisted with microbial control in the pre-RO equipment. Most RO membranes are extremely sensitive to miniscule amounts of chlorine making its removal of significant concern. In addition, although not shown, pH adjustment may be required, or desirable, prior to an RO to improve overall performance. The RO produces permeate, or product water, and a reject or waste stream. Permeate is then treated by a continuous electro-deionization (CEDI) module that further improves the water's chemical quality. Since CEDI technology does not purport to control microorganisms, it is common to place a sterilizing grade filter (0.2 or 0.22 μm) after the CEDI module and prior to the distillation unit. In addition, although not shown, an optional ultraviolet sanitizer may also be present prior to the final filter to reduce the number, and likelihood viable organisms will populate the filter surface and eventually pass through it. Used in combination, UV prior to filtration typically lengthens the usable bacteria-retentive life of the filter. The final step in creating WFI using this configuration is a single-effect or a multieffect distillation unit from which WFI is supplied to a storage tank for distribution throughout the facility (Fig. 3).

The design depicted in Figure 2 is generally regarded as one of the more simple conceptual arrangements based on the inclusion of limited unit operations prior to distillation.

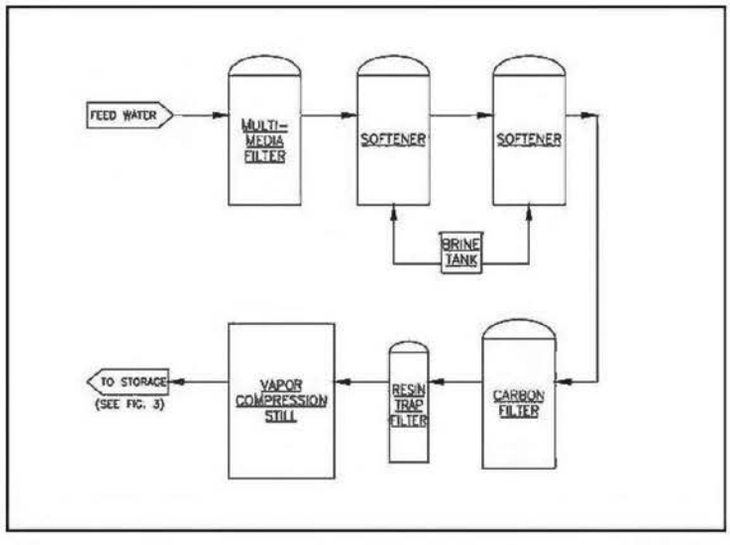

Figure 2 Schematic of a typical vapor compression still application with softened water feed.

The ability to limit pretreatment is driven primarily by the selection of a vapor compression distiller, which is less likely to become fouled by certain ionic constituents in the feed water than a traditional distiller, on the basis of operating temperature. However, it should be recognized that this equipment may be associated with a cost premium possibly based on purchase price and/or operating cost. Hence, like the previous example, the savings associated with the purchase and operation of less complex pretreatment may be partially offset against increased capital and/or operating cost associated with a more complex still design.

These simpler WFI system designs (Fig. 2) include only basic pretreatment, usually consisting of back-washable media filtration followed by dual series water softeners, similar to that of the previous example used for pretreatment prior to a traditional distiller. The media filter, as in the previous example, is used to remove suspended contaminants, common in potable water, in the size range of 10 μm and larger. The subsequent softeners are resin-based exchange type devices used to remove hardness, including calcium, magnesium, etc., to minimize the potential for downstream scaling of the still. The softeners in both examples exchange hardness for sodium ions that are less likely to impact subsequent downstream components. If hardness were not removed, components such as the RO and still would experience the residue buildup similar to that occurs on residential plumbing fixtures such as sinks, tubs, and showers. Again a post-softener cartridge filter is commonly included for removal of any resin fines that might be generated as the resin degrades and to protect in the event of a catastrophic resin release resulting from softener failure. Unlike the first example, RO and CEDI are usually not required to protect a VC distiller; however, this is confirmed during feed water analysis.

An important situation that cannot be overlooked relates to chlorine and/or chloramines that are commonly present in the feed water as part of a sanitization regime implemented by a municipality (or other supplier). Chlorine has been used extensively since the early 1900s; however, the carcinogenic effects of resulting trihalomethane by-products and other issues have resulted in increased use of chloramine (primarily chlorine and ammonia) by municipalities and other organizations for sanitization of potable water. It should be further noted that chloramines may be more difficult to remove than free chlorine. Chlorine, especially at the elevated temperatures within a still, will result in corrosive attack of the stainless steel construction hence it must be removed prior to distillation. Whereas the chlorine in the first example is addressed by sodium sulfite injection this example includes granular activated carbon (GAC). However if chloramine is present, it should be noted that ammonia is a by-product of chloramine removal in a GAC, which is not removed during distillation. Hence positioning of the GAC upstream of softening eliminates this concern as ammonia will be removed by softening, although at the expense of dechlorinated water in the softener. Softener bed capacity must be carefully considered when ammonia removal is important based on ion removal order relative to hardness and ammonia. The addition of a GAC (regardless of placement) makes the system slightly more complex and must be considered during capital and operational cost comparison/evaluation.

Product water from the still is again fed to a distribution storage tank which in turn feeds distribution sites throughout the manufacturing area(s) where WFI is further processed or used for formulation, cleaning, etc. This distribution gives rise to additional significant concerns relating to both chemical and microbial quality maintenance and will be discussed further below.

The design examples presented above do not include schematics for distribution, as distribution for either design would probably be very similar for the same application. Notwithstanding, distribution is a critical part of any bulk system design as it requires its own specialized features and poses its own challenges to the designer. Not only must it comply with all the appropriate good practices, but it must be able to blend into the building structure seamlessly to avoid conflicts with functional utilization including process and occupancy-related issues. Distribution design often makes it very difficult to interface easily with other utilities such as heating, ventilation, and air conditioning in a three-dimensional setting such that the cubic volume of the facility is not unreasonably reduced or compromised. Distribution must efficiently and effectively deliver suitable quality water at appropriate pressure and temperature and in volumes commensurate with manufacturing or process requirements.

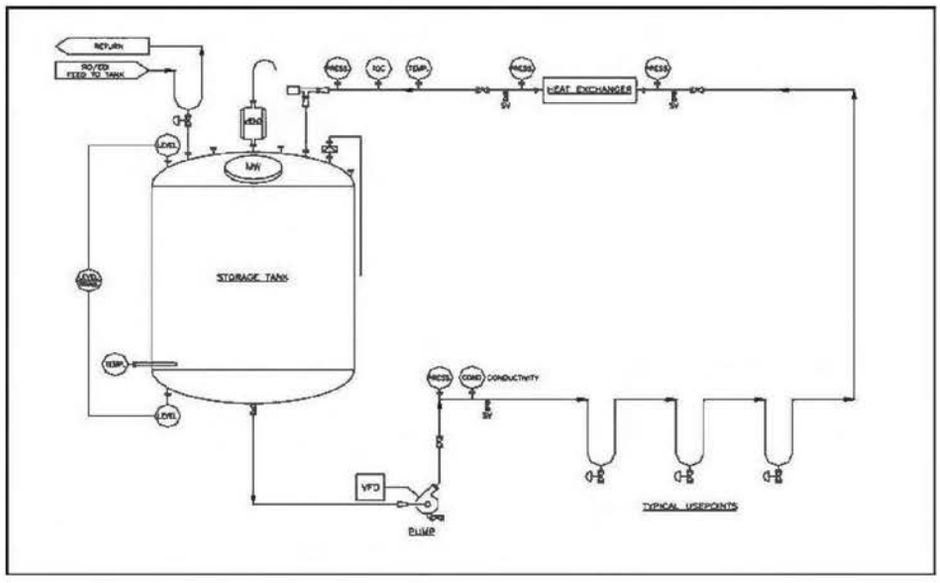

Figure 3 Schematic of a typical WFI distribution system.

Distribution must allow for monitoring of the water so that quality attributes can be ensured and as well practical controls must be available to operations personnel to avoid the use of water that does not conform to necessary requirements. This necessitates the inclusion of monitoring devices and instruments suitable for determining the water quality, comparing those attributes to an accepted value and controlling various features or operations to ensure unsuitable water quality is not delivered for use in production-related activities. Certain components must be strategically located for access and for ease of service and calibration while minimizing downtime that would result in lost productivity. A typical WFI distribution schematic is shown above in Figure 3. It is important to recognize that typical distribution systems contain no provisions for correcting WFI deficiencies (retreating the water) hence the distribution must be configured such that it does not reduce water quality below acceptable levels either as the result of poor design or because operation and/or maintenance is inappropriate.

Schematically, certain unique features in a pharmaceutical water system may be readily apparent. For example, user points are not shown as branches from a main header as is common in traditional industrial, residential, or commercial piping systems. They are represented as typically installed in a WFI system where the piping is looped and water recirculates as close to the actual delivery point as possible with continuously flowing water, often only fractions of an inch away from the actual point of utilization (see Fig. 3). This practice reduces the potential for water stagnation but significantly increases both the size and complexity of most WFI distribution systems based on increased pipe length and often as a result of increased pipe diameter. Other unique aspects of WFI distribution include complete drainablility based on pipe pitch, targeted flow velocities, and specialized materials, connectors, and finishes as well as design that is suitable for sanitization.

Some of these "best practice" aspects of water system design, including materials and finish, will be discussed further. Flow velocity will be partially addressed adjunct to the following biofilm discussion; however, it should be noted that target design velocities range from above five feet per second (FPS), measured directly as velocity to simple establishment of turbulent flow based on a calculated Reynolds Number. The use of Reynolds Numbers, a dimensionless numeric, has recently become popular in spite of the lack of agreement as to exactly what value represents turbulent flow (figures often used range from 2500 5000).

Notwithstanding, flow velocities using Reynolds Numbers result in significantly lower system flow rates than the previously applied velocities ranging from 3 to 5 FPS. For example, a 3" OD sanitary distribution network designed for 5 FPS velocity results in water flow exceeding 101 GPM. Alternatively, use of a Reynolds Number of 3000 results in water velocity of less than 0.136 FPS and flow of 2.7 GPM; more than a 35-fold variation in design flows.

In spite of these changing trends, designers must consider all facets of good design to ensure the piping remains flooded, is capable of sanitization and minimizes biofilm formation, all while not adversely affecting water quality, either chemically or microbiologically, and while remaining capable of delivering proper water volumes, temperature, and pressures as required. Good piping practices usually dictate the inclusion of pressure gauges, sample valves and isolation valves before and after each major system component to enable rapid diagnosis and remediation of any system anomalies that occur.

Sampling can also have a profound effect on distribution system design based on facility design and operation as it is commonly accepted that water used in manufacture should be sampled in the same fashion as it is used. Hence, piping that is direct connected to vessels, tanks, or equipment poses an additional challenge for sampling that must be overcome. Sampling for process control will often result in procedures that are different than those used as part of a quality program related to ensuring products meet requisite standards and specifications.

Best practices for sanitization design are also the subject of debate; however, it is never inappropriate to include the flexibility to utilize multiple methods of sanitization in the event one method fails to deliver acceptable results or alternative methods are required. The primary methods of water system sanitization include periodic or continuous application of heat using hot water or steam (most popular for WFI Systems), periodic chemical sanitization (less favorable), and the use of ozone where the main volume of stored water is continuously ozonated and the distribution network is periodically ozonated (not currently applied to WFI). It should be noted that WFI systems designed primarily for heat sanitization can also be sanitized with chemicals including ozone provided the design is appropriate and proper controls are implemented to assure no residual that could adversely affect WFI quality remains after completion. Both chemicals and ozone can also be implemented on an ad-hoc basis in suitable locations provided the system hardware is capable of contact with these materials.

Heat sanitization is regarded as the most reliable as heat is able to penetrate through even a substantial biofilm; however, heat is typically unable to remove the dead biomass leaving a readily colonizable nutrient rich base for future generations of organisms. Hence, the use of supplemental methods may be required as systems age and biofilm develops and evolves.

Finally, the issue of system drainability is often misunderstood as the reasons for draining can vary significantly. In some systems, draining occurs on the basis of intermittent use and the speed, and ease of draining results in lower cost and higher process reliability. Alternatively, systems that undergo periodic sanitization with chemicals must be drained to remove residual sanitant with drainability again lowering cost and improving reliability. However, WFI systems that are steam sanitized require drainability to assure removal of steam condensate that may be below sanitization temperature. Trapped condensate within the system may compromise the sanitization effectiveness by creating "cold spots" that are not fully sanitized and can result in system microbial recontamination or rebound based on system operational characteristics. For systems that operate continuously heated, the requirement for drainability is less critical as only during periods of outage for service or maintenance does drainability become a concern.

As previously indicated, in addition to the primary (pretreatment) and secondary (distillation) treatment steps to bring the water quality to acceptable levels, other important functions must be performed, such as maintaining the quality of the water after it is produced and testing to ensure compliance. These additional requirements increase the complexity of any bulk system dramatically. Maintaining large volumes of water to precise chemical and microbiologic standards can be very difficult as contact with air, piping, equipment, etc. will result in rapid deterioration of the water quality. As a result, specially designed components, expensive materials, and costly processes are usually required to ensure maintenance of the water quality.

For example, WFI is usually maintained at 165°F (equivalent to approximately 74°C) or even higher to reduce microbial proliferation and to maintain suitable microbiologic control. This method of bacteria control is costly from both an operating perspective and because it limits the materials of construction, increasing the system capital cost. Designs usually include a recirculated loop piping configuration, which helps to maintain temperature, for the purpose of minimizing bacteria and biofilm-related problems, but with a resulting cost increase associated with greater energy use, increased piping length and size, and higher installation costs. These and many other specific design practices associated with WFI systems increase complexity, cost, and maintenance, and unfortunately, few options are currently available to the designer to eliminate or even reduce these common "good practice" requirements.

Costs associated with WFI systems are extremely high when compared to comparable commercial or industrial systems of similar size. This is not the result of a single expensive component or technology but rather is the result of a multitude of contributing factors that build incrementally on each other. For example, valves cannot just be stainless steel (SS), nor can they be simply 316SS, they must be 316LSS. In addition, they cannot have just sanitary end fittings; they must be sanitary throughout often including areas of nonproduct contact. Furthermore, they must be certified with support documentation that traces each subcomponent to its original source, including verifications of manufacture, finish, and any other pertinent details. These requirements are necessary for virtually every component in the system driving the cost as a result. As well, the installation of WFI systems usually involves a more highly skilled work force and the use of specialized tools is often mandated to assure the quality of the completed installation.

In addition, it is commonplace for WFI systems to be constructed of sanitary stainless steel beginning with portions of the still and including components such as vessels, pumps, valves, instruments, and all other WFI contact components. Sanitary materials typically include special ends for joining components that are more hygienic than industrial joining methods such as threads or flanges. These components are usually polished to finishes of 32 μin. roughness average (Ra) or better with some designers specifying better than 10 μin. Ra finishes, supplemented by electrochemical polishing to obtain mirrorlike reflective surface conditions. Sanitary piping is, in actuality, not piping at all but rather sanitary tubing. Piping is simply the generic reference term used based on common parlance. In simple terms, pipe is industrial quality cylindrical material used to transport many fluids. It is measured nominally and designated by its interior diameter such that 1 in. diameter pipe has an inner diameter of approximately 1". Conversely, based primarily on the precision required for polishing, sanitary tubing is used for WFI applications and indeed for most sanitary applications. Sanitary tubing that is designated as 1" in diameter has an actual exterior diameter of 1" as opposed to the approximate inner diameter used to measure pipe. Sanitary fittings are typically fabricated from the same precision tubing to ensure exacting alignment during assembly, which further results in smooth interior surfaces that are cleanable and drainable with fewer large gaps into which microbial growth can extend.

Stainless steel finishing itself is an extensive subject and is beyond the scope that can be effectively covered within this chapter. However, it should be noted that most finishes are mechanically applied, progressively, using increasingly finer abrasives, similar in concept to that used for wood sanding. Higher quality finishes typically require application of electrochemical polishing, or electropolishing usually over a high quality mechanical finish. The reverse of plating, electropolishing, also known as chemical machining, removes surface material to create an extremely smooth and attractive finish that may be easier to clean based on the materials in contact with the surface. In addition, electropolishing creates a passive layer on the surface of the stainless steel, increasing its corrosion resistance. Passivation will be discussed in more detail below.

Another aspect of WFI system complexity is that of the specialized welding required, which serves to reduce the number of mechanical joints and as a result reduces maintenance costs and minimizes sites for possible leaks. Unlike the traditional welding methods with which most of us are familiar (manual welder wearing a welding mask or shield as protection from an exposed arc), the process employed for WFI piping is automated and typically enclosed. The computer controlled welding power source works in conjunction with a

precision automated welding torch that encloses the area around the weld joint. The equipment precisely controls all facets of the welding process, including voltage, current, rotational speed, arc gap, and time, at a level unmatched by a manual operation. When supplemented by specialized pipe cutting and end preparation tools, proper procedures and inspection equipment, virtually flawless conditions can be repetitively achieved.

Orbital welding, as this process is known, removes the variability associated with manual welding but requires skilled labor that is trained in all facets of sanitary installation to ensure system integrity. The cost of the welding equipment is substantial, far higher than similar industrial machinery; however, the welding machine is only a portion of the complement of related tools needed to complete the work. Additional specialized tools are usually required such as precision cutting and facing machines, gas analyzers, tungsten grinders, and specialized inspection devices used to view the interior of the tube after it has been welded. These ancillary components can easily exceed the cost of the welding system by two to four times.

Inspection devices, also known as borescopes or videoscopes, are similar to the endoscopes used by medical professionals. A miniature video camera is attached to the end of a fiber-optic cable that in turn is attached to a video processor with recording capability. The scope can be inserted into the tube, prior to installation, to confirm the interior finish and can also be used to view the completed weld for conformance to the specification. These devices are often in the range of 25 ft in length, based on the length of standard tube sections, and to negotiate bends (elbows or tees) many scopes are equipped with articulation. Articulated movement allows the operator to navigate sections of installed tube with minimal potential for damaging the interior finish.

It is also noteworthy that continuous quality monitoring has become the norm for WFI production relying heavily on electronic instrumentation and controls that are typically integrated into the system. These devices, although typically not mandated by regulation, allow for more consistent and reliable quality tracking and may in the long run be more cost-effective than other alternatives. Continuous monitoring can be accomplished for a significant number of attributes including conductivity, TOC, temperature, pressure, flow, and level using in-line, at-line, or on-line equipment. Other data important for ensuring the proper operation of individual unit operations may be necessary or desirable. Hence, it is not uncommon to find pH, chlorine, oxidation reduction potential (ORP), and ultraviolet intensity monitors as part of the water treatment monitoring regimen, or even to find dissolved ozone monitors as part of a feed water sanitization program. Unfortunately, instrumentation for continuous microbial monitoring (enumeration, detection and/or speciation) is not currently available although a number of rapid microbial detection systems have evolved that can speed the traditional and laborious work associated with pour plates, incubation, and colony counting. Laser detection systems, based on light scattering technology, currently in development and testing and may eventually be capable of performing these functions and may prove to be viable options in the future. Interestingly, process analytical technology (PAT) was in use for pharmaceutical water systems long before the FDA's risk-based initiative with PAT was implemented.

MICROBIAL CONSIDERATIONS AND SANITIZATION

It has been said that the chemical purification of water is by far the easier part of producing WFI, while the microbial control aspect is far more difficult. The reason for this is primarily because the technologies used to chemically purify water are well known and understood, proven through years of application and use, and are mechanically and operationally reliable. Coupled with limited sources for chemical recontamination that are relatively easy to control, the chemical purity of water can be readily achieved and maintained. Alternatively, microbial contamination is mostly unseen, monitored using random grab-samples that are typically not representative of actual conditions and often misunderstood by engineers responsible for water system design. Organisms can react to their environments, such that many can survive in low-nutrient environments and under stressful conditions. Organisms can exist as planktonic entities floating unprotected in a water stream, and they also have the ability to attach themselves to surfaces, no matter how smooth, in search of nutrition. Attachment results in the creation of a biofilm that serves to protect and insulate the organism from sanitants

while providing a relatively nutrient-rich environment in which reproduction can safely occur and from which further colonization can originate.

Initially organism attachment to a surface is relatively weak; however, once stationary (sessile), the microbe quickly begins to produce a sticky polymeric material known as glycocalyx, which more firmly attaches the organism to the surface and helps it adapt to sessile existence. This conversion will occur fairly rapidly, primarily dependent on the species present and the level of nutrients, such that transition can take hours in some cases and days in others. As reproduction occurs, additional strongly attached cells develop forming a community that provides further protection and benefits for the occupants as the sticky extracellular glycocalyx snags floating nutrient particles, other planktonic organisms, and even flocs of biofilm that may have come loose and become free-floating.

Much like the water itself, biofilm development is seldom similar from site to site. The biomass that develops is based on the nutrients present and the affinity or adaptability individual species have for that available nutrient base. Biofilm will be somewhat the product of local conditions; for example, in stagnant or slow-moving water, biofilm will tend to grow taller and further into the water stream exposing greater surface area. However, this growth is far more susceptible to shear forces that might occur during water use, sampling, flushing, or as the result of other system events such as water hammer. Alternatively, in fast-flowing turbulent water biofilm will tend to be denser, with less exposed surface that would be susceptible to disturbance from the action of the water movement. Biofilm provides a secure stationary base for organisms and is not directly monitored by traditional sampling. The results of such water sampling typically only represent a small portion of the actual microbial content in a system. In other words, traditional sampling is based on "grab" samples of water rather than sampling of the resident biomass. The reason for this dichotomy may be the result of misguided thinking or may be reaction to the difficulties associated with more suitable sampling techniques and test methods. However, regardless of the reason, the fact is that far more organisms are likely to be resident in biofilm than floating in the water stream. This will be true of most systems except those that are under continuous sanitization conditions such as systems that are operated at elevated temperatures (heated) constantly. Various rationales have been offered for the current testing methodologies, with the only appropriate justification based on a continuously sanitizing environment, where biofilm would expect to be virtually nonexistent as a result of the hostile conditions.

In fact, biofilm development in most systems undoubtedly complicates sampling methods such as might be the case when dense tightly adhered biofilm results in few, if any, planktonic counts during routine testing, incorrectly interpreted as very low levels of microbial activity. Other anomalies in the same system may additionally confuse and cloud analysis, when sudden and unpredictable release of sections or flocs of biofilm into a sample indicate significant counts far above those seen during routine tests.

Excellent and concise summaries of water system microbiology have been written by T.C. Soli; as part of *Microbiology in Pharmaceutical Manufacturing* (8) and for the upcoming revision to the *ISPE Water and Steam Baseline Guide* expected to be released in 2010, subject to FDA review and final approval. These summaries provide nonmicrobiologists with a clearly written, simple, down-to-earth explanation of microbial concerns relative to water systems written by an expert with years of practical experience in pharmaceutical manufacturing, consulting, and as a member and vice chair of the USP Expert Committee on Pharmaceutical Waters.

As noted, heat is the sanitization method most commonly employed during WFI production, storage, and distribution for microbial control. Those wishing to either employ RO or to operate their systems below 65°C must address difficult issues, such as the limited ability of RO membranes and the most common RO equipment to operate hot continuously. As well there is a perceived regulatory expectation to dump unused WFI after 24 hours if it is not heated above 65°C for sanitization.

Other common methods of sanitization that may be appropriate for less critical applications are usually not deemed acceptable for WFI. However, industry convention and regulatory interpretation that has banned the use of ozone for sanitization of WFI, even though it has become common for Purified Water application, is currently being reevaluated, and

changes may ultimately result. If this is indeed the case, the cost of producing WFI will most likely drop, based primarily on energy savings and supported further by "green" initiatives and "carbon footprint" reduction.

Previous concern relating to ozone use was based on the dictate in the WFI monograph that precludes added substances. In the past, this had been inappropriately interpreted to mean that no chemicals or materials could be added to water destined to become WFI. This has since been clarified so that it is understood that any substance added to the water to facilitate its treatment must be removed prior to use and that adequate monitoring and documentation is required for confirmation of its removal. On the basis of ozone monitoring limitations, controversy still remains regarding whether detection limits for ozone are adequate to ensure WFI safety. There has also been previous concern since earlier, now superseded, USP language existed stating that distillation (or RO if approved) must be the final treatment step to produce WFI. Hence, since ozone is a chemical oxidizing agent that would be added to WFI, after production, for the express purpose of maintaining biologic control; many believe that ozone addition would not meet the intent of having distillation as the final process step. These issues are currently the subject of many industry discussion groups as they try to come to grips with drug manufacturing cost and the FDA's Pharmaceutical cGMP's for the 21st Century: A Risk-Based Approach, and PAT initiatives.

MATERIALS OF CONSTRUCTION

The primary material of construction for the vast majority of WFI systems is stainless steel of the 300 series, typically 316SS. This austenitic alloy is resistant to rusting and many other forms of corrosion associated with water applications provided chlorine and chlorides are not present. Stainless steel is generally considered to be corrosion resistant and easy to fabricate. It can be polished to present a uniform, smooth, and pleasant-looking reflective surface that is considered cleanable and hygienic. Stainless steel is relatively inexpensive when compared to more exotic alloys and is readily available in the 316L alloy configuration most suitable and accepted. When produced and utilized in this low carbon "L" grade version (316LSS), it is additionally resistant to forms of corrosion that may occur as a result of field construction such as that which might be required to build or assemble a WFI system within a pharmaceutical manufacturing facility, making 316LSS by far the material of choice for WFI-related applications.

Stainless steel is a unique alloy with iron as the predominant component, yet it is corrosion and rust resistant based primarily on the alloying constituents added that instill its special properties. The mechanism that makes stainless steel suitable where other iron-based alloys would fail is termed passivity. Passivity is a naturally occurring surface oxide resulting from chromium used as an alloying material. The chrome oxide layer that develops is extremely thin, typically in the range of 5 to 50 Å (1 Å equals 1 ten-billionth of a meter) yet, except for extremely corrosive environments (including chloride attack), this very thin covering is adequate to protect the material from many common corrodants.

The passive layer that protects stainless from corrosion forms in contact with air and can easily be disturbed during the manufacturing process (by tools and abrasives), during the installation process (by welding and handling), and during use (as a result of high-temperature operation, high flow velocity, and due to the chemically aggressive nature of WFI). Hence, it is common for materials, equipment, and systems to undergo passivation procedures to expedite and enhance naturally occurring passivity. These procedures can take any number of forms; however the goal is to recreate or strengthen the natural passive layer and to reduce the time required before the material is suitable for use. Passivation or repassivation procedures typically involve either submerging the parts or, as may be the case for large systems, filling the components and recirculating the required solutions, both for a suitable time and at a suitable temperature to achieve the desired result. Procedures usually include a caustic cleaning step to remove oils or other contaminants, followed by contact with an acidic solution to remove surface iron. Rinsing with purified water ensures no residual chemicals remain, which might impact either the stainless or the WFI. This procedure allows the passive layer to form more quickly and to be more robust.

Finishing or polishing of stainless steel, as discussed in the preceding text, is often a hotly debated topic relative to pharmaceutical application. Some believe that the smoother surface results in slower and less tenacious biofilm development; however, this is not supported by microbiologists who believe that even the smoothest finishes available only minimally delay biofilm development and have minimal impact on organism attachment. Notwithstanding, most pharmaceutical and biopharmaceutical equipment receives some degree of polish to improve the appearance and ostensibly to improve cleanability. It is commonly agreed that large-scale surface imperfections either in material surfaces or at connection points afford microbes a safer haven from sanitants and hence should be avoided. The disagreement typically resides in the definition of "large" such that one school of thought is to avoid crevices larger than the organism itself, dictating extremely smooth surfaces, while the alternative posits that attachment will occur regardless of the surface finish inferring that only minimal surface preparation is needed. Both schools of thought agree that minimizing mechanical joints is prudent and where necessary the use of sanitary connections is recommended.

TECHNOLOGIES SUITABLE FOR PRODUCTION OF WFI

As mentioned, there are a limited number of production options currently available for WFI. For example, in certain regions of the world, RO technology is permitted for use in producing WFI. However, as noted, only the use of distillation is permitted by the EP for WFI production. Hence, any firm wishing to employ RO to make their WFI will find it either more challenging or even impossible where regulations insist that alternatives to distillation be proven technologically for each and every application.

When produced by distillation, there are two primary technologies used for the manufacture of WFI. The first is traditional distillation wherein an evaporator and a condenser are connected in series to first evaporate the feed water to steam, leaving behind any contaminants, and then to condense the pure steam that has formed to water of WFI quality. This simplified explanation is intended to offer the reader only a conceptual understanding of a far more complex process that requires separation of contaminants from the pure steam and will include either the use of rising film or falling film evaporation, as well as other technologies appropriate to the design.

A variation of this design includes the connection of multiple evaporators to increase efficiency. These additional evaporators or "effects" use the pure steam that is produced to generate additional capacity, thus reclaiming energy that might otherwise be wasted. Units ranging from 3 to 6 effects are common based on utility steam pressure/temperature; however stills with 7 or more effects are possible although often the savings cannot offset the added cost of the required equipment. It is critical to note that this process, termed "multieffect" distillation, is not a multidistillation process as the water is evaporated to steam and condensed only once, no matter how many effects are employed (Fig. 4). Traditional distillation typically involves no moving parts (with the exception of valves, etc.) and is driven most often by utility steam although electric and superheated water driven units are possible. Product from this type of distiller is typically at/or above 185°F and near ambient (or atmospheric) pressure.

Traditional multiple effect distillers typically require greater levels of pretreatment, owing to the higher operating temperature; however, this higher temperature is an advantage when it comes to pyrogen destruction.

Alternatively, VC distillation is a technology that includes an electrically driven compressor within the still to increase the initial pure steam pressure (by 1 3 psig) and temperature and the resulting higher-energy steam is used to generate additional capacity (Fig. 5). This still technology is typically configured to produce WFI between 80 and 85°C but can produce water at lower temperatures more efficiently than traditional distillation with outlet temperatures for "cold" WFI normally 6 to 15°C above the feed water temperature. Noncondensable gases are of greater concern for VC stills and as a result most are equipped with either a feed water deaerator (decarbonator) or a vent condenser. VC distillation, however, typically requires significant amounts of electricity to power the compressor, which partially offsets the lower steam consumption common for this type of equipment. As well, the compressor adds a level of mechanical complexity and is a source of added maintenance beyond that required for traditional distillation.

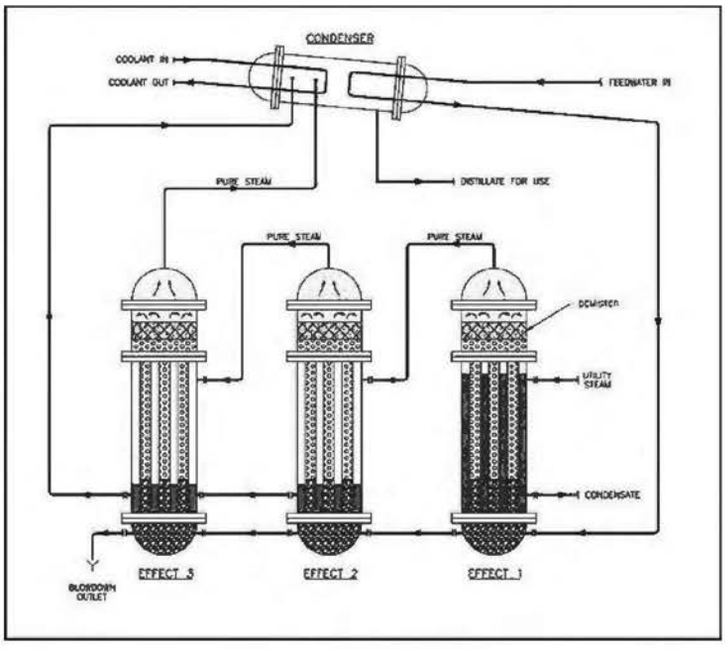

Figure 4 Schematic of traditional distiller (multieffect type).

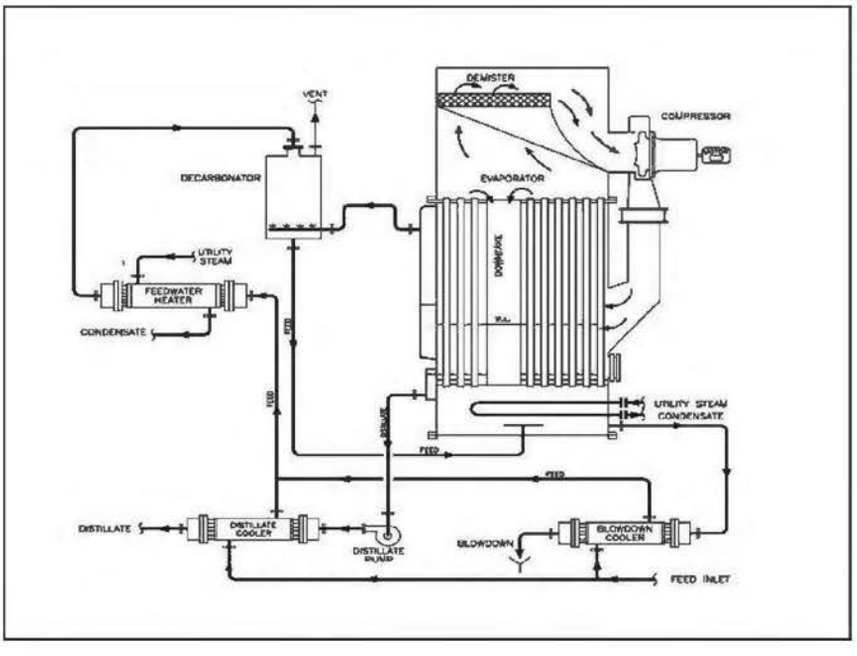

Figure 5 Schematic of vapor compression distiller.

For either distiller, design is critical such that steam velocity through the unit cannot allow entrainment of water droplets that would carry contaminants along with the steam into the final WFI. Therefore, mist eliminators/separators and other options including centrifugal force are usually employed to ensure final WFI quality and suitability. For both unit designs, water in the evaporator section requires periodic purging to eliminate the concentration of contaminants that result from the continuous evaporation. This process is termed "blow-down," and is initiated typically by the equipment controls (usually at a fixed rate) based primarily on the volume of WFI produced, but may also activate during start-up and/or shut-down of the equipment. Silica scale is of concern for all stills and the rate of blowdown is one mechanism used for its control. Stills are typically capable of at least a 4 $\log^{10}$ (99.99%) reduction in endotoxin making it imperative that feed water is of suitable quality so as to not over tax the unit's capability.

Instrumentation and controls for either design must be adequate and capable of ensuring the quality of WFI produced. Any control failure could result in contaminated WFI with the associated rejection of product, production delays, and possible patient injury if undetected. Overall still quality, including design, materials, finishes, components, and assembly, is imperative to WFI quality, as is the requirement for reasonable and sufficient distiller maintenance. Monitors and controls within the WFI distribution are also required to ensure that the WFI quality has not been compromised and that delivery system integrity is uncompromised.

Alternative technologies can be implemented to produce WFI when not constrained by regulation or practicality. These options may include RO and ultrafiltration (UF) that are both based on the use of a barrier to limit the passage of contaminants. RO, which is commonly used for producing Purified Water, employs a semipermeable membrane capable of passing water molecules but which does not allow the passage of contaminants that are typically larger in size (Fig. 6). This pressure driven tangential flow filter works in reverse of normal osmosis, hence the name. RO equipment produces a continuous waste stream of water, when in operation, that is typically in the range of 15% to 30% of the influent. This "reject" water continually flushes the membrane surface removing contaminants that would otherwise clog the membrane. The waste stream can amount to a significant volume of water, discarded as waste to a local sewer, and often necessitates creative thinking to develop alternative uses, including cooling tower or boiler makeup, vessel prerinsing, lawn sprinklers, etc. Product water, also termed permeate, from a single pass RO is usually unable to meet the conductivity requirements of Purified Water and is often supplemented by subsequent deionization, possibly in the form of CEDI, to ensure that the water produced is of adequate chemical quality. Since deionization is considered by most regulatory agencies as unacceptable for the final treatment of WFI, those considering RO as a means of production should only consider double pass (2-pass) RO, also known as product-staged RO, which must be operated meticulously, possibly with optional features, to ensure chemically suitable effluent quality on a continuous operating basis.

RO membranes are capable of the finest filtration commercially available with removal rates typically below 1000 molecular weight (Daltons) and often specified with a molecular weight cutoff (MWCO) of approximately 100, meaning molecules of 100 molecular weight or greater are rejected at greater than 90%. Recent developments allow for some membranes to be heat sanitized, providing a significant improvement over previous products that experienced the drawbacks associated with chemical sanitization. New style membranes, capable of continuous operation at or near 185°F, are becoming commercially more viable, eliminating the possible downstream growth that has plagued RO technology for decades because of the inability of chemicals to sanitize the permeate side of the membrane.

UF is capable of particulate removal typically between 10,000 and 300,000 Da, and is not nearly as fine as RO membranes. However, UF under certain circumstances may have the

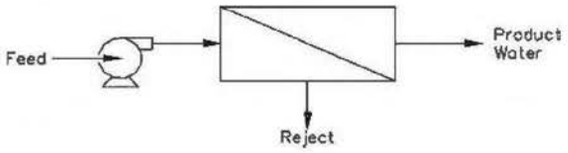

Figure 6 Schematic of reverse osmosis.

ability to operate at significantly lower pressures than RO such that UFs can be utilized at pressures more common to WFI systems and without special high-pressure ratings. Whereas the majority of RO membranes are sheet material in a spiral wound configuration, UFs can be constructed in alternate forms, including hollow fiber and in various materials such as porous solid ceramic, making some more readily sanitizable. Although UF technology may offer options in the future, these designs are currently the least accepted for manufacture of WFI, since UF technology is only specifically listed and approved by name for WFI production in the current Japanese Pharmacopeia (9) and no other compendia.

There are no definitive published statistics regarding WFI production methodologies; however, it is estimated that more than 98% of the WFI created worldwide is made using distillation of one type or another, with the remaining, less than 2%, made using alternate technologies such as RO and UF. In fact, this author was unable to confirm even one system officially validated for WFI manufacture using UF technology anywhere in the world.

Once produced, WFI must be protected against contamination and maintained at suitable quality levels to ensure its safety. Accomplishing this task is far harder than often realized and some system designs have been flawed because inexperienced designers failed to recognize the rigors associated with WFI storage and distribution. Storing of WFI at/or above 165°F is acknowledged to be the most secure and robust method; however even simple related tasks such as delivering the water to manufacturing or packaging sites within a facility can prove extremely challenging. For example, piping that might be used to carry the WFI to its point of intended use could become problematic based on cooling below sanitization temperature if use were not continuous. As a result WFI piping is almost always sanitary stainless steel, recirculated as close to use-points as possible and continuously reheated to ensure a suitable temperature is maintained. There can be no deadlegs at lab or work benches, sinks, or equipment that would cool sufficiently to allow microbial growth. Dead-legs, piping sections that cannot be circulated, of even short lengths often result in contamination that can disable an entire WFI system. Dead-legs can occur as the result of closed valves, improper piping techniques, or improperly mounted accessories such as instruments. Dead-legs are a continuing topic of debate relative to tolerable length. Suffice it to say that dead-legs should be minimized as opposed to application of a maximum length "rule." Common industrial or commercial piping practices cannot be employed in WFI systems as they would undoubtedly result in long-term system quality issues based on their nonsanitary nature.

WFI piping that operates at an elevated temperature will commonly require insulation to reduce heat loss, as well as for personnel protection and to reduce operating expense. Suitable insulation must be chloride free to reduce the risk of corroding the stainless steel piping system and components. Insulation should be continuous to avoid cold spots that might harbor bacteria. This situation results in a conundrum since continuous insulation would hamper the maintenance, service, and calibration efforts that are required to maintain the system in good operating condition and in a validated state.

WFI distribution systems often must traverse hundreds, if not thousands of feet of distance within a manufacturing facility to service users in varied locations. This piping often rises up or drops down through multiple floors within a building. High flow rates, compounded by the need for recirculation, often result in the requirement for relatively large diameter piping. This piping within a facility may transit through unconditioned spaces such as attics or on roofs or through minimally conditioned spaces such as ceilings or warehouses. These situations compound the difficulty of maintaining a system's integrity and pose additional challenges relative to temperature maintenance and as a result may also influence sanitization efforts and validation.

Stainless steel in the 300 series, although extremely corrosion resistant, is susceptible in water systems to a phenomenon termed "rouging" where deposits of metal oxides (mostly of iron-based origin) form on the surface of the stainless steel materials and eventually migrate throughout the entire system. This condition is most prevalent in WFI systems at temperatures in the range of 165 to 185°F (or higher, as in the distiller) and becomes progressively worse as water purity and/or temperatures increase. This situation has been documented extensively over the years at virtually all facilities operating within these parameters, although the degree of rouge that develops is often inconsistent from site to site.

Three primary types of rouge have been defined and cataloged by Tverberg (10) of which all are frequently found in pharmaceutical and biopharmaceutical environments. Interestingly, rouge that develops typically does not affect water quality based on current standards, resulting in many operators ignoring rouge as they consider it to be irrelevant. However, rouge that continues unchecked can result in particulate generation, which is certainly unacceptable in WFI. Also, in extreme cases excessive rouge results in pitting of the base metal that damages the surface finish reducing the hygienic effect and can, in severe cases, result in leaks around seals and gaskets.

Rouge can be orange-red in color and loosely adhered such that it could be removed by wiping with a clean soft cloth. This type of rouge may develop quickly in elevated temperature systems or more slowly in systems operating at lower temperatures. Rouge can also occur in darker shades (red-brown or bluish), which are usually more tightly attached as the result of higher temperatures or extended periods. Both of these rouge types are common in WFI systems and can usually be removed by chemical treatment. Unfortunately, even after removal, it is common for the condition to recur requiring periodic derouging and repassivation of most WFI systems that operate at or above sanitization temperatures. Rouge is often first detected in areas of high velocity such as in pump volutes and spray devices presumably the result of erosive action and/or microcavitation.

A third type of rouge is more common to systems operating above 212°F with characteristic dark blue-black color that is so tightly adhered that removal may damage the base metal making it unsuitable for continued use. This type of rouge is most common in Pure Steam systems or WFI systems that are regularly/frequently steam sanitized.

Derouging of water systems can be performed using various procedures; however, passivation chemicals are not typically effective for rouge removal and the effectiveness of a passivation procedure is often compromised if rouge is present and not removed prior. On the basis of the variety of rouge types, it is prudent to design equipment and systems with removable/replaceable coupons (a fitting or small tube section is sufficient) that can be utilized off-line to test a proposed derouging procedure.

Because of the critical nature of the effluent produced by a WFI system, validation, or more recently commissioning and qualification, activities are critical to system acceptance. As well, ongoing acceptance is predicated upon suitable system operation and upkeep. Effectively, all components must be maintained properly and must operate as intended, such that the system must produce WFI consistently. Any operational trends that provide indication that performance is deteriorating must be addressed and control reestablished to ensure water quality is not out of specification.

SUMMARY

It is surprising to many that water deemed acceptable for human consumption, and termed potable, requires significant additional treatment to allow its use in drug manufacture. As a minimum, filtration and softening are commonly employed as pretreatment and when required, additional unit operations are included as necessary prior to distillation to ensure water quality and system reliability. In addition, microbial control is required throughout the entire process impacting the selection of components, their arrangement, and method of operation. As well, sanitants and sanitization procedures cannot be universally applied on the basis of compatibility with individual unit operations overall system suitability.

WFI, whether injected into a patient directly or as part of a parenteral preparation, must meet exacting standards. Chemical and biologic quality must be unquestionable as many patients that receive treatment are already in a compromised state such that any quality imperfection might prove to be detrimental.

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5 | Particulate matter: subvisible

D. Scott Aldrich

OVERVIEW

Definitions

Pharmaceutical Dose Forms

United States Pharmacopeia (1) defines the following parenteral dose forms:

a. *Injection* Liquid preparations that are drug substances or solutions thereof,
b. *For injection* Dry solids that, upon the addition of suitable vehicles, yield solutions conforming in all respects to the requirements for *injections*,
c. *Injectable emulsion* Liquid preparations of drug substances dissolved or dispersed in a suitable emulsion medium,
d. *Injectable suspension* Liquid preparations of solids suspended in a suitable liquid medium, and
e. *For injectable suspension* Dry solids that, upon the addition of suitable vehicles, yield preparations conforming in all respects to the requirements for *injectable suspensions*

This chapter will address the presence and nature of subvisible particulate matter in regard to these dosage forms, and in a general aspect for any medical product.

Particulate Matter

The primary subjects of this chapter are particles and their size. The following discussion explores the lack of specificity for these subjects; to be comprehensive in our evaluation, we must not consider only particles that are one entity or characteristic, nor may visibility be described by a single "line-in-the-sand" size threshold or definition. In practice, the ultimate definition(s) of particulate matter must be suitably broad to allow us to consider many different aspects of character and size to make the products we develop most robust. Our discussion concerns particles and specifically, those we are unable to see. Our ability or inability to see them is not a simple matter, and dependent on many factors. Overall, our intent is to find the particle(s), understand them, and change something to alleviate their presence, inclusion, growth, or change. But first let us discuss the primary topics.

Particulate Matter—What Is It?

Particle: a body having finite mass and internal structure; a minute portion, piece, fragment, or amount; a tiny or very small bit; a grain, speck

Particulate matter (PM) is a broad term to include many varieties of conditions, sizes, and associations of particles in the product fluid. In an ideal state, a single particle is a single type of material, in solid form present in the pharmaceutical product and detected by a human observer or an electronic counting device. Generally, if detected by human vision, it is visible. However, certain attributes of the PM may yield "visibility" of material when in a size far below human resolution. Human detection of PM is probabilistic, not deterministic. PM is not an intended component of the formulation, such as active ingredient or suspension agents. The current USP definition (2) is *"particulate matter in injections and parenteral infusions consists of mobile undissolved particles, other than gas bubbles, unintentionally present in the solutions."* The intent of this definition is interpreted by some as warning to exclude only extraneous matter or contamination from the final product.

Just what is contamination? Is it the presence of unwanted and foreign material in the product? Is it a noxious substance that may cause harm, such as microbial colonies, spores, pyrogens, chemical substances extraneous to the formulation, particles of vermin (flies, insects, etc.), environmental debris, package fragments? Is it a form or derivation of the active ingredient or excipient mix that has now appeared? Yes to all. Some are certainly worse than

others and cannot be tolerated, such as filth (insect parts) and pyrogens. But that should not be the limit of our concern. Other PM, while not contamination in a filth sense, is deleterious to the physical integrity of the formulation.

A more comprehensive and quality-relevant definition of PM is to include all forms of nonformulation substances that may be seen or detected by analytical means, at release and for the shelf life of the product. Careful and comprehensive investigation of the PM content, type, and origin is the only means to true high-quality products in this category.

Certainly, PM is an unintended consequence of product manufacture. Only extraneous forms should be unexpected, that is, PM forms that may arise from within the product are well understood and excluded. In the worst case, PM appears after batch release and while the product is being distributed, stored, and used. PM may cause further problems such as formulation change, appearance, dose performance change, dose delivery effects, and medical impact on the patient. The pharmaceutical product is expected to be an example of careful planning and utmost control. Occurrence of PM and even worse, unexpected change in the nature of the formulation or package because of its presence is a failure. Thus, USP Chapter <1> Injections definition of foreign and PM uses *visible particulates* to define all, so as not to discriminate between types or allow one category over another (3).

Particle Categories

PM implies an ideal; a solid or collection of solids, observed as a single solid. Solid PM or a collection of solids are certainly the most prevalent nature of PM; however, many other and alternate conditions (nonideal) may be considered as PM by the observer, and may affect the quality of the formulation. If we are to comprehensively explore the content of PM in the final package, we must consider the following as well:

- Immiscible liquids
- Immiscible semisolids
- Microscopic solids, in sufficient number to produce observable light scatter(haze)
- Microscopic solids joined by a matrix to form larger entities
- Thin solids, even in large sizes, invisible to normal observation (require high-intensity light and reflectance necessary for visualization)
- Poorly dissolving product, from dry powder or lyophilization reconstitution
- Package-attached material, of any size
- Combinations of any one or more of the above, and with single solids

Particulate Matter Size

Visible: The greatest distance at which a person with normal eyesight can see and identify an object. Or, the smallest object (characteristic) a person may readily detect with unmagnified 20/20 vision.

Particle size: x:y:z dimensional size in micrometers, Feret's diameter, Martin's diameter, chord, longest dimension, geometric volume mean, projected area diameter (equivalent circular diameter), area.

Size is an absolute measure, described in three dimensions. The x, y, and z axes of the solid entity describe its volume, are the basis of its shape, or habit, and provide a seemingly boundless number of descriptive terminology. The size of a sphere can be reported unambiguously by radius or diameter. Cubic or otherwise equant ($x \sim y \sim z$) particles may also be well described by edge length or diameter. However, consider other measures, such as minimum length, maximum length, same sedimentation rate, volumes, areas, weight. Which categorical measure would we use and report? Just as for determinations of powder character in regard to fine, coarse, large, and small, the method and analysis one employs affect the outcome and must be relevant to the industry.

When thinking of PM in terms of a (single) dimension or size, the correlation of x-y-z dimensionality to a single factor or equivalency can be confusing. For spheres, we can state a single and relevant dimension, diameter. No matter what orientation, we still observe a size of diameter. For all other (and more common) particle shapes, which dimension shall we use? Correlating the subvisible population to a single index or area under the curve from a range of

Table 1 Sizes of Common Materials

Common material	Sizes
Flu virus	0.07 μm
Pollen	7 100 μm
Diameter of human hair	50 150 μm
Sneeze particles	10 300 μm
General size descriptions	
Molecular	<1 nm
Colloidal	1 nm 0.5 μm (protein formulators consider >500 nm to be coarse)
Floc or flocculate	Size: sub 10 μm, but more importantly as character: suspended, aggregated material held together by weak physical forces such as surface tension or adsorption
Fines	up to 10 μm
Powder, particles	up to 100 μm
Coarse	up to 500 μm
Granular	>500 μm
Chunk	Millimeters
Mass	Really big!

Source: From Ref. 7.

the particle population would be another form of limits test, but has not been generally accepted (4,5). Particle load in the product and subsequent impact on the patient, especially in chronic administration of medication, is quite important. Setting the appropriate limits within constraints of manufacturing and across all product forms is not accomplished by a single threshold size or load limit (6). The emphasis is one of continual product improvement to ensure patient safety and robust final packaged product.

Some common particle types and sizes are shown in Table 1.

What truly is subvisible? There are a number of conflicting reports for the lower threshold of visibility, ranging from ~11 to ~150 μm. Delly (8) reported an absolute minimum detection of 11 μm on the basis of the minimum arc of view at reasonable near vision, given the optical lens system of the human eye. Literature often (9,10) defines 30 to 50 μm as the distinction dimension between the visible size for human visual inspection and truly subvisible PM size. Review of visual inspection threshold at the 1995 PDA meeting (11) reported 150 μm as a reasonable, probabilistic threshold of detection (70%) for trained inspectors in defined commercial release assays for a single particle in a clear liquid formulation, within a transparent uncolored glass ampoule. On the other end of the size range, the fundamental range for protein formulations is defined as those particles too large for size-exclusion chromatography (>0.1 μm), yet below visibility (<100 μm) and for protein active ingredient, the 0.1 to 10 μm size range is quite important for monitoring formulation stability (12). The size domain below 10 μm is not addressed by the current compendia.

You see from these examples disagreement from established and reliable scientists determining visibility dimension in a variety of applications. I believe they are all correct, in the given application and at the point in time of measurement. I will not set a specific, hard threshold for the subvisible zone because of a number of factors. One must consider and accept the concept that the visible dimension and subvisible dimensions meet in a gray zone of detection, one of probability not certainty. We can agree that a 10 μm particle would be very difficult to observe visually, whereas, a 300 μm particle should be seen quite readily under reasonable inspection conditions.

Certainly, the training of the observer, the method of detection, and the attributes of the PM affect visibility. Consider the properties of PM in Table 2 that may enhance or diminish detection. Greater visibility of PM in the fill solution is given by increased size, distinctive color, extremes of buoyancy, and high reflectivity. These properties also enhance the visibility of small particles that would otherwise go unnoticed. Further, the significance of the PM in the pharmaceutical product has not so much to do with its size and hence visibility, but its effect or potential effect on the quality and integrity of the medicinal agent. Pharmaceutical aerosols are

Table 2 Properties of Particulate Matter

	Detection probability	
Property	Enhanced	Diminished
Size	>100 μm	<100 μm
Spatiality	In foreground	In background
Color	Bold	Weak, pastel
Contrast with matrix	High	Low
Reflectivity	High	Low, none
Buoyancy	Neutral	Extremes

preferentially 2 to 5 μm (13). Pharmaceutical suspensions are formulated in near micrometer to ~30 μm crystals, dependent on the requirements of the API. Microsuspensions and nanoparticle formulations are by definition submicrometer in nominal size. What is the material, how is it intended to be included in the formulation or formed, what is its stability, and what is its nature? While many of the above descriptive categories relate to dispersed powders and dry solids, it is important to use consistent and common terminology for description.

PM is a cause of instability in the pharmaceutical liquid and is signaled by observation of PM or change in the filled product package, by excessive PM load determined by quantitative assay and by an upward trend of PM content even if below compendial limit. USP and major compendia sets limits for subvisible particles, dependent on container nominal fill volume, at ≥10 μm and ≥25 μm size thresholds, certainly below the lower end of the visible range (14).

PARTICULATE MATTER CHARACTER

Particulate Matter Category

Extrinsic: outside, from the exterior
Intrinsic: inherent, part of the whole

An important concept, discussed in many reviews of PM, is the categorical origin of particulate matter. Two general categories describe all sources; first, *extrinsic* or truly foreign matter, introduced during batch fabrication (formulation assembly, package preparation, or filling operations) and resident in the package. This added particle load does not change unless due to fragmentation, swelling, (hydration), or degradation. The second, *intrinsic* particulate matter derives from product-related sources such as formulation-container incompatibility, component impurities, formulation degradation, substance extraction in points of contact, component precipitation, nucleation, sedimentation, etc. All are likely to change over time and may not be detected until months after release. These categorical types cover all particle populations.

Particulate Matter Nature

Table 3 shows the nature of the particulate matter as the state or degree of complexity, the fundamental identity of the substance(s). We consider the extremes: from simplest (single crystal) to the most complex [multiple species, varying crystallinity and combination of physical state (liquid and solid with entrained gas)]. These conditions provide the boundary of PM nature and from simplest to complex, least to most challenge for analytical detection and characterization. Both extremes may occur in the developing formulation and may cause deleterious effect. Are they detected and identified soon enough to be removed prior to late-stage trials?

Table 4 shows crystallinity states; considerations, which may apply to all, a portion, or in a continuum state of the solid particle. All of these properties may be used to identify the material.

Certain terms are the basis for common descriptions and are important to understand for subsequent identification of the unknown material, a shown in Table 5.

Table 3 Common Associations of Particulate Matter

- Singular
  - Liquid
  - Solid
  - Combinations
- Multiple
  - Aggregate/agglomerate
    - Boundaries (matrix evident?)
    - No boundaries
    - With similar material, foreign material
    - Groups of groups
    - Homogeneous heterogeneity
  - Crystal grouping
    - Polycrystalline many large individual crystals
    - Microcrystalline many small individual crystals
    - Cryptocrystalline many nucleation sites apparent, or their effects evident, without resolution of discrete particles
- Layered, banded, lamellar
- Coated
- Foliated platy aggregate
- Suboptimal and continuum states

Table 4 Crystallinity States

Nonevident Amorphous, glassy
Evident or continuum
- Liquid crystals: 2D order (mobile, yet ordered)
  - Nematic parallel molecules with at least one rotation axis: thread or cable like arrangement
  - Smectic molecules arranged in layers, with long axis perpendicular to the layers (planes)
  - Cholesteric nematic layers in a helical arrangement
- Solid crystals: 3D order
  - Isometric (1 ref. ind.)
    - Three equivalent axes, intersect at right angles
- Uniaxial (2 ref. ind.)
- Tetragonal
  - Three axes, two of which identical in length, intersecting at right angle. Third axis is longer aligned at right angle to others.
- Hexagonal (trigonal)
  - Four axes, three of which lie in the same horizontal plane, at 120° angle. Fourth axis is perpendicular to the plane formed by the others, and of different length
  - Biaxial (3 ref. in)
- Orthorhombic
  - Three axes of different lengths, intersecting at right angles.
- Monoclinic
  - Three axes of different lengths intersecting such that a and c form an oblique angle, and b is perpendicular to the plane formed by the other two.
- Triclinic
  - Three axes of different lengths all intersecting at oblique angles.

Table 5 Common Descriptive Terminology

1. Clustered observed as a group, but without bonds or matrices holding the PM together
2. Aggregated particles touching, joined at edges, fragile collection easily broken apart
3. Agglomerated particles joined at faces, fused, in a tenacious collection; not easily broken apart per ASTM (15). Others note that all assemblages are agglomerates and that an aggregate must be confined to prenucleation association of molecules that may crystallize (16).
4. Cemented particles held in a solid matrix by another material, such as stones in a cement sidewalk.
5. Inclusions particles, liquid, or gas entrained in another solid or semisolid
6. Occlusions PM held on the exterior of a solid or semisolid, such as "sugar on a doughnut."
7. Material is *sectile* if it sections into plates.
8. Spherulitic three dimensional, radial association of material; a ball of needles all with one end in a nucleus. Also called a rosette.
9. Splintered fragmented by force into thin pieces, not necessarily with consistent dimension or habit, which would indicate crystalline subunits.

Table 6 Appearance Shapes/Habit and Axial Ratios

Name	Description
Equant	$x \sim y \sim z$ are roughly equivalent; cube, sphere
Rod	$x \sim y < 3$ and z: up to 50
Column	Thicker rod $x \sim y$: 2 6 and z: 10 50
Acicular/needle	Very thin rod $x \sim y < 1$: $z > 10$, often >100
Plate	x: 7 10; y: 1 5; z: 7 10
Flake	Thinner plate x: 7 10; y: <1; z: 7 10
Blade	x: 7 10; y: 3 5; z: 10 50
Lath	Thinner blade x: 7 10; y: <3; z: >10
Ribbon	Thinner lath x: 5; y: <1; z: >50

Table 7 Luster and Hardness Scales

Luster (relectivity: 10 = high)	Mohs hardness scale (10 = high)
Absorbing 0	Talc 1
Earthy 1	Gypsum 2
	Calcite 3
Silky 4	Fluorite 4
Pearly 5	Apatite 5
Greasy 6	Orthoclase 6
Waxy 7	Quartz 7
Vitreous (glassy) 8	Topaz 8
Flash 9	Corundum 9
Adamantine 10	Diamond 10

Appearance

Descriptive terms for the particle exterior may offer insight to the formation and exposure. Shape or habit description of the solid may seem difficult at first, but in reality, only three basic shapes need consideration, with subsets of dimensional variation. The boundaries of the axial dimensions may be arguable, but need to be defined and then consistently followed or best communication. These are shown in Table 6. Opacity of the material may be described opaque, translucent, transparent, and possibly variations thereof. Upon close examination, surface characteristics may also offer insight to identity, formation, exposure, and condition.

In the collection of material properties, one cannot overlook *luster*, a reflective property that may indicate PM origin or even identity and is often correlated to hardness. Note the series of hardness values with representative minerals, and the correlated luster examples shown in Table 7 (17).

Common Particulate Matter Types

The common types of PM encountered in pharmaceutical injections, primarily from earlier and less-refined manufacturing designs that allowed too much of the natural world to creep into the liquid fill, was first addressed by Australian researchers Garvan and Gunner (18,19). This impact is well documented in the literature (20 24) and discussed in topical lectures (25,26). Table 8 contains a listing of prevalent categories. While less common in modern parenteral products, contamination by natural materials is always a threat to the sterility and purity of the product.

The commonality of particle types has much to do with the sources and ability of the assembly and filling arena to exclude them. These types are (*i*) the commercial assembly equipment (metals, polymers, resins); (*ii*) assembly arena design and use (airborne and personnel-borne PM pollens, molds, bacteria, epithelial cells, hair, fibers, soil minerals, insects); (*iii*) packaging (olefins, glass, silicones, rubber) and processing (silicones, lubricants, phthalates); and (*iv*) formulation components. Thus, a more comprehensive and categorical listing is appropriate.

Table 8 Common Particle Types

Filth	Common extraneous matter	Intrinsic matter
Insect parts	Glass	Crystalline material
Bacteria	Rubber	Diatoms
Fungi	Metal	Extracts
Biological debris	Paper and cellulose	Leachates
Human epithelial cells	Starch	Drug changes
	Talc	Package degradation
	Facility contributions	Process contributions

Minerals and inorganic species such as titanium dioxide, iron oxide, zinc oxide, calcium sulfate (carbon black), clays, calcite, etc., added intrinsically from septum systems and environmentally from soil and the processing arenas.

Fibers and fragments:
Cellulose in all forms, natural and processed cotton, rayon, wood, paper, clothing dust.
Man made nylon, polyester, Aramide, olefins, Teflon, Nylon, Orlon, Lycra, etc.

Hairs: Human, anima

Metals: Primarily stainless varieties; however, the effects of dissolved metallic contamination from excipients, active ingredient, formulation processing, and storage are significant. Minimal content of Al, S, Ca, K, Ba, Cr, Fe, Ni, Cu, Zn, V, Sn, Ag, and others may be significant enough to induce a cascade of change on the otherwise stable dissolved active. Instability of β-lactam antibiotics cascades from leached divalent ions from plastic and glass containers, rubber stoppers, and tubing. In particular, leached Zn catalyzes the opening of the β-lactam ring leading to API degradation and insolubility and thus nucleation of subvisible particles (27).

Biological matter: Seeds, trichomes, pollens, vegetation fragments (cell wall, structural components, leaves), and insect fragments.

Processing: Extracted organic moieties such as phthalates, emulsifiers, silicones.

Minerals: (*i*) filler components of septum (closure) and other modified elastomeric systems and (*ii*) local and trafficked soil-related minerals not removed during preparation and added during filling.

Building: Paint and components (titanium dioxide, calcite, mica), gypsum (wallboard), calcite, rust, polymers, epoxies.

PARTICULATE MATTER SOURCES

Formulation/Package, Manufacturing, Administration Practices

All points of contact, residence, manipulation, and storage may contribute to the particulate matter load in the final product. A large but not exhaustive list of these sources includes:

1. Manufacture of the bulk active ingredient (processing hardware-extracted moieties, nanoparticle content), all of which will pass through finishing and sterilization media;
2. Sterile manufacturing operations (residence in sterilized stainless vessels, air exposure, HVAC quality, personnel, transfer tubing/filters/gaskets exposure), sterilization media extractable moieties;
3. Filling arena stainless exposure, valves, gaskets, tubing, in-line filters,
4. Container cleanliness and residence;
5. Pharmacy operations of withdrawal/transfer, reconstitution/transfer, exposure to IV sets, dilution with and in sterile vehicles (D5W, normal saline, Ringer's solution, etc.), temporary receiving-transfer vessels; and
6. Container use contributions. These include piercing of the septum, connection of IV sets, activation of sliding septae, multiple product chamber mixing, needle quality and handling.

In review of the literature, one is drawn to the conclusion that much of the gross and obvious sources of particulate matter have been identified, verified, and removed from the systems and packaging that accommodates parenteral medications. This is generally true for established companies and their legacy product processes in which constant analytical vigilance is maintained for particle content and variation. Much is known about the entire process stream, and strict control is imparted for microbiological, chemical, and physical insult.

The trending of batch results and facility performance for well-maintained visual inspection and subvisible particulate matter assays is the key operation for controlling particulate matter content. Maintenance of these operations is an important modifier, since all may be run as discrete operations, without proper quality control, for example, use of targets and action limits that precede specification performance; investigation of variation and out-of-target results to identification of rejection or out-of-limit, incident cause, and remediation. Connection of the detection and quantitation assay units with the organizational units responsible for batch investigation is an important process improvement factor.

However, in consideration of new facility operations, new product lines, new equipment, site changes, component vendor alteration or new vendor, new package, new therapeutic line to name a few, increased particulate matter inclusion in the final product is more likely until these parameters are understood and controlled.

PARTICLE DETECTION

Human Inspection

No discussion of subvisible particulate matter is complete without consideration of visible particles, for two reasons:

a. The division between visible and subvisible is not a hard line at a single particle size.
b. Visible and subvisible particles are bound together regardless of size (at the moment) since there is often a relationship if not origin from time of fill to final shelf age.

The USP defines visible particulate matter to consist of mobile undissolved particles, other than gas bubbles, unintentionally present in the solutions. This definition has been interpreted by some as meaning extrinsic or foreign material only. This is not true since the methods detect all forms of solid matter. We should add *. . .that may originate from extrinsic sources or from changes due to instability of the product and in some instances may be seen as immobile due to storage conditions or aging.*

Visual inspection provides an effective means of evaluating the physical integrity of product candidates and is required, in some manner, by all major compendial organizations for final package presentation. The pristine appearance and high integrity of the product is the result of a complex R&D effort to limit particulate matter load in the final package, contributed or formed from active or excipient ingredient, formulation pH/tonicity/concentration, container quality and cleaning and the manufacturing processes to assemble the final product package. The change of particle load or presence of visible "particles" in the final package necessitates their removal from the batch or, if not detected, could cause recall of the distributed product.

Typical Visible Particle Pharmaceutical Tests

Appearance Test Under Ambient Condition

The most prevalent means of inspecting a parenteral container, and any retail object, for that matter, is visual examination with normal vision in ambient light. Now, to give the object a good scrutiny, one takes a little time to look on and through the package in good lighting (500 1000 lux) with normal reasonable vision (at least 20 ft/40 ft where 20 ft/20 ft is standard visual acuity). Background is not an important issue, just a reasonable examination in good lighting. We should see obvious package problems such as label type and placement (presence?), fill volume, color and gross condition, package integrity (intact, cracks, soiling?). A few seconds duration suffices to observe these conditions, depending on the level of scrutiny and familiarity with the package. This is an obvious step, not often used within the laboratory.

Visual Inspection in Light Box

Compendial procedures ordain the use of a final visual inspection step to release parenteral packages. All units exhibiting particulate matter, package defects, or cosmetic anomalies must be rejected and culled from the batch. Pace, light intensity, and background are all controlled and important assay parameters, with maintenance of trained and adept inspectors within a quality assurance program also necessary. It is in this application that we experience "what is visible?" Much variation of opinion is seen in the literature, with many definitions of 50 μm as lower detection limit. However, the true detection limit depends on many factors, including number and type of particles, package characteristics, method of inspection, and capability of the inspectors; however, one can rely on the compilation by Parenteral Drug Association (28) for visual inspection detection of one particle in a simple package (clear solution, glass ampoule), as 1% probability of detection at 50 μm, 50% probability of detection at 100 μm, and 70% probability of detection at 150 μm. The importance of analytical particle detection and counting to cover the region below 200 μm is obvious.

Visual Inspection with Tyndall Beam Light in Light Box

A strong, collimated light beam will scatter when encountering dispersed, small solids a Tyndall effect. The size of the solids that will yield scatter effects ranges from colloidal (500 nm) to about one micrometer. Above that, one may observe "twinkling" reflection from individual, otherwise invisible particles. Use of qualitative Tyndall inspection is insufficient alone, being a complementary method for over-lighting inspection, revealing settled solids and otherwise invisible but large populations of submicron particles.

EP 2.2.1 Clarity and Degree of Opalescence of Liquids

Nephelometry as a measure of haze or cloudiness per *EP 2.2.1* is a comprehensive and quantitative development tool. While not a requirement in U.S. formulations, it is a prudent measure of formulation stability and acceptability. Often, it is not the absolute measure of formulation clarity that is important, but the variation unit-unit. Comparison to four levels of haze allows grading of the parenteral formulation.

PARTICLE QUANTITATION

Particle Quantitation Methods—Compendial

Light Obscuration and Membrane Filtration Microscopic Quantitation Methods

PM quantitation for discrete size thresholds may be accomplished by many methods, such as electrozone, light blockage, light diffraction, light scattering, and by filtration of the liquid and counting of the retained solids. All are quantitative, sensitive, and linear, with low variation and are easily calibrated. Light obscuration (LO) methods are preferable in one sense, for all the reasons one uses controlled instrumentation, and large populations of particles are sampled. Controlled and repetitive testing may be performed with a minimum of time, cost, expenditure. Trending of pharmaceutical liquid particle populations is routine and preferred by most analytical groups. The method offers defined instrumental parameters, calibration, and good correlation between labs with minimal variation. USP maintains a particle count reference standard (PCRS) and solicits participation from industrial, academic, and government labs to validate each generation of the standard. The USP promotes collaboration of pharmaceutical users and subsequent consensus on calibration approach.

However, LO is not preferable in one way: it is nondiagnostic (one does not visualize or capture the particles causing counts), is prone to artifact counting (air, immiscible oils), and size derivation is dependent on the nature of the particle. Instrumental methods provide some vector or derivation of true size, for example, equivalent circular diameter (ECD), geometric volume mean, or chord for example. For a spherical particle, the diameter will very closely equal the reported size, but for particles with nonequivalent axes ($x = y \neq z$) such as a rod or flake, the reported size is much different than the actual dimensions one would view or measure microscopically.

Membrane microscopy (MM) is the secondary or Tier 2 method endorsed by USP for subvisible particulate matter. Although microscopy is a preferable diagnostic tool, its

application is more time-intensive and tedious, may be more subjective in particle size evaluation, and not as well known, thus the method is not commonly used for routine release or stability assay. However, in a well-controlled laboratory with experienced microscopists, the membrane isolation and microscopical counting method provides diagnostic insight to the level and type of particulate matter occurring in the stability batch. Are the particles a variety of types, or more indicative of a single, or point source? Are there singular or aggregated, amorphous or crystalline, package or formulation particles present? Is the particle load low or trending higher? These are all good questions and necessary for process improvement. One cannot pursue the elimination of particulate matter without an understanding of their origin(s). Recent harmonization of these two methods among the USP, Ph. Eur., and JP compendia has occurred (29). The tests for subvisible particle content may be used interchangeably in the ICH regions, given suitable instrument calibration and system suitability requirement satisfaction.

The two-stage determination of particulate matter load for these products utilizes the LO method for routine lot release and stability evaluation, supplemented by the historical membrane microscopic method. The current methods have been revised from previous compendial procedures and the revised particle content limits official in USP XXV. LO (light extinction, light blockage) electronic particle count methodology is the desired stage I method, using tabulation from electronic detection of light blocked by particulate matter in the sample fluid streaming past a fixed photodiode detector.

The membrane method is provided for particle counting by microscopical examination of a membrane isolate, as a stage II method. The membrane microscopic method was described as "referee" by some researchers due to retention and examination of the particles after counting. One can reexamine the results, to determine the nature of the particles, and verify the count. LO samples are lost, or if captured during testing are at least corrupted and may not be useful in a retrospective analysis. The LO method is certainly more reliable in regard to standardization and precision; however, it will suffer influence from immiscible liquids, air, and particles with nonequant (1:1:1) habit. Both methods have their attributes and drawbacks, and are best used in conjunction with other analytical equipment configured for small particle analysis.

In the process of conventional microscopical particle sizing, the operator utilizes a calibrated linear scale to compare particle dimensions and render judgment according to desired criteria1. The USP graticule design, Figure 1, provides a defined "graticule field of

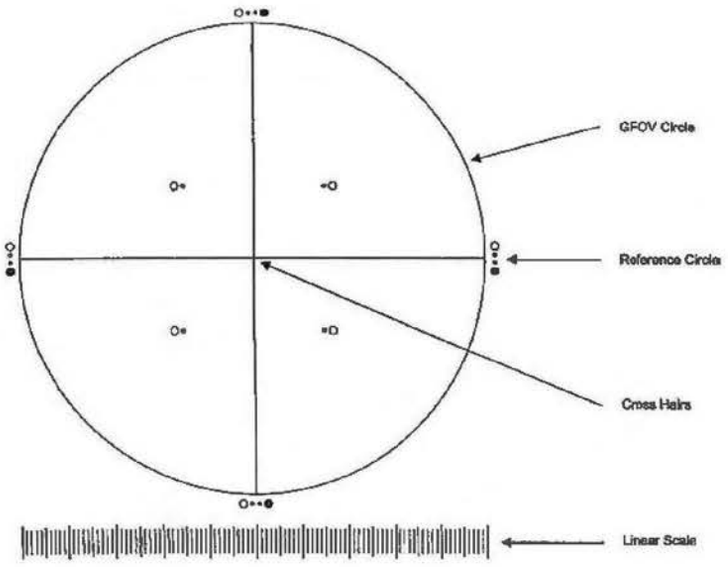

Figure 1 USP graticule. Reprinted with permission. Copyright 2009 United States Pharmacopeial Convention. All rights reserved.

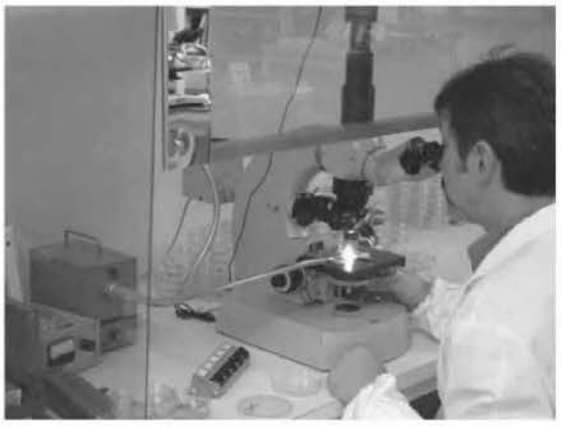

Figure 2 Operator examining a membrane isolate within an UAFW.

view," comparison circles and a linear scale. The design facilitates the estimation of particle size on the membrane through comparison to open and filled circular areas with 10 and 25 μm diameters or to the linear scale in 10 μm divisions. The operator, Figure 2, scans the entire filtration area of the membrane, determining size against the calibrated scales and tabulation particle counts in the threshold bins.

The tests contained in USP Chapter <788> are physical limits tests performed for the purpose of counting or enumerating subvisible extraneous particles within specific size ranges. There is also a guideline for liquid ophthalmic products, USP Chapter <789>, that follows the chapters <1> and <788> direction. USP Chapter <788> testing applies to the following dose forms: all large-volume injections for single-dose infusion (large IV bag or bottle) and small-volume injections (smaller vials and ampoules), unless otherwise specified in an individual compendial monograph.

USP Chapter <788> is now harmonized with the Japanese Pharmacopeia and European Pharmacopeia (EP), providing the test approach for counting particles by two methods and relevant PM limits. The injectable product is first tested by the LO procedure or method 1, having a specific set of particle content limits. If the test article fails to meet the limits, it may be tested by and must pass a second method, known as the membrane microscopic procedure, also referred to as method 2, with its own set of limits. The limits are defined dependent on parenteral container volume and method used: count per mL for large volume injectable products (1.A. LO and 2.A. MM, >100 mL) and per container for small volume injectable (SVI) products (1.B. LO and 2.B. MM, 100 mL and less) as shown in Table 9. For nominal 100 mL volumes, the 1.A and 2.A. limits are used in the United States.

There may be technical reasons the injection product cannot be tested by LO, and so microscopic testing may be used exclusively. Documentation will be necessary by the company to demonstrate that the LO procedure is incapable of testing the injection or produces invalid results. Finally, while it is expected that most products will meet the requirements on the basis of the LO test alone, it may be necessary to also test some by both methods to (*i*) prepare for any future use of the membrane microscopic method and (*ii*) reach a conclusion on conformance to requirements. An example may be a formulation that consistently yields

Table 9 USP Chapter <788> Particulate Matter Limits for Parenteral Products

	Method 1 light obscuration		Method 2 membrane microscope	
Parenteral volume	≥10 μm	≥25 μm	≥10 μm	≥25 μm
A: LVI: >100 mL	25/mL	3/mL	12/mL	2/mL
B: SVI: 100 mL and lower	6000/container	600/container	3000/container	300/container

higher counts by LO, and the membrane method verifies lower counts, possibly due to air or immiscible liquid artifacts.

The membrane one selects for the MM assay may be any porosity 1.0 μm and less, gray-black-color contrast; however, in practice the 0.45 μm and less presents a much more flat and homogeneous background. Avoid gridded membrane varieties. They have marginal value in when partial counting and just get in the way when scanning across the membrane.

Chapter <788> states "... do not attempt to size or enumerate amorphous, semiliquid, or otherwise morphologically indistinct materials that have the appearance of a stain or discoloration on the membrane surface." These materials will show little or no surface relief and present a gelatinous or film-like appearance. The historical precedent for this caveat was specifically for large volume parenteral diluent products, especially terminally sterilized varieties that contained a small amount of degraded, gelatinous material in the fill. In development of this method, participating LVP firms producing terminally sterilized dextrose solutions encountered degradation products that were nondiscrete particles, consisting of 5-hydroxymethyl furfural, a known impurity in the products (30). Reactions of proteins with 5-HMF and the presence of further breakdown products such as levulinic acid and so-called "humins" all contribute to the browning of the solutions and presence of gelatinous material (31). If excluding all gelatinous substances during the count, conducting and interpreting the count results will clearly reflect only solid particles; however interpreting the final solution quality may be difficult. If any gelatinous material or any material eluding particle definition is a persistent isolate, this sort of retained material may be indicative of product change or instability and should be investigated during development. It is prudent to investigate the consistent presence of an unknown and uncontrolled material.

High molecular weight and proteinaceous formulations are prone to active ingredient aggregates, ensembles, semisolids, casts and skins that seem ever-present and not rinsed away. In development, it is prudent to investigate these materials, especially when observed only occasionally, as they may indicate a system or condition out of control. If a particle appears to have three dimensions as viewed under the microscope, then it is a particle. However, when using membrane methodology in development, if persistent or significant forms are not classified to be countable particles, it is necessary to pursue their identity and cause to avoid lot failure later.

There is a counter-intuitive part of the size determination in MM method 2 for classic analytical microscopy. In the process of conventional microscopical particle sizing, the operator utilizes a calibrated linear scale to compare particle dimensions and render judgment according to desired criteria1. The USP graticule design provides a defined "graticule field of view," comparison circles and a linear scale. The design facilitates the estimation of particle size on the membrane through comparison to open and filled circular areas with 10 and 25 μm diameters, or to the linear scale in 10 μm divisions.

One does not need to directly superimpose circle or scale over the particle, but can estimate particle size in the field of view by comparison to the circles on the graticule. USP Chapter <788> states ... "transforming mentally the image of each particle into a circle." Thus, rather than using the more direct and simple longest dimension or maximum chord, the intent of the committee and the revision to "Improved Microscopical Assay" (IMA), the particles are to be counted after classifying them in regard to an ECD. Barber (24) provides the historical reference for this conversion, with work by the HIMA USP advisory committee (32). This research revised the original MM assay and provided the basis to fit microscopical counts to the instrumental count paradigm. One converts the area of nonspherical particles into the area of an equivalent circle. This is a problematic conversion, since it can only be performed "mentally" and by visual comparison to the 10 and 25 μm circular features. Without image analysis and accurate calculation of the observed area, one must process this decision on an estimate of the diameter or radius and convert to area. Since area $= \pi r^2$, the shape of the particle can be converted to area by deriving the radius from the x-y dimensions of the resident particle. If we first find the equivalent radius (ER) of the x-y dimensions by the square root of $(X \times Y) \div 2$, then equivalent circle diameter $= \pi(ER)^2$. This conversion is the basis for the mental conversion of the observed particle to an area and would bring the count of nonspherical particles more in line with that produced by the LO assay. Mentally converting the actual size of the visualized particle into an equivalent size category generated by the

Table 10 Comparison of Axial Dimensions to Calculated Equivalent Circular Diameter and Resultant USP <788> Category

Table Size. Conversion of particle habit size to equivalent circle diameter

Shape	*x* axis	*y* axis	*z* axis	ECD	ED	USP size
Sphere	10 μm	10 μm	10 μm	79 μm^2	10 μm	≥10 μm <25 μm
	25 μm	25 μm	25 μm	491 μm^2	25 μm	≥25 μm
Equant	30 μm	35 μm	25 μm	824 μm^2	32 μm	≥25 μm
Flake	10 μm	10 μm	4 μm	79 μm^2	10 μm	≥10 μm <25 μm
Rod	50 μm	5 μm	5 μm	196 μm^2	16 μm	≥10 μm <25 μm
	150 μm	2 μm	1 μm	236 μm^2	17 μm	≥10 μm <25 μm
	200 μm	4 μm	4 μm	626 μm^2	28 μm	≥25 μm
Needle	70 μm	1 μm	1 μm	55 μm^2	8 μm	No (<10 μm)
	200 μm	1 μm	1 μm	157 μm^2	14 μm	≥10 μm <25 μm
	300 μm	3 μm	1 μm	707 μm^2	30 μm	≥25 μm

ER = equivalent radius = square root of $(X \times Y) \div 2$
ED = equivalent diameter = $ER \times 2$
ECD = equivalent circular diameter = $\pi(ER)^2$

preferred analysis is quite cumbersome when confronted with highly aspherical particles. Considering the reference USP circles, the 10 μm circular area is 78.5 μm^2 and the 25 μm circular area is 490.9 μm^2. Observed particle area would be judged against these comparators, shown in Table 10. The comparison is quite revealing; roughly spherical or equant, flake, and tablet-shaped particles will be sized quite directly against the linear scale or the circles, since the two largest axes present nearly equivalent circle area dimension. For particles with an elongated shape axis, such as long laths, rods, fibers, or needles, however, where one or two axes are quite minor, the estimate of size according to an equivalent circular area will yield significantly different values from longest dimension.

Thus, rod and needle shapes, while quite long, would be counted in much smaller categories (if at all) than the apparent lengths. Many companies have converted the maximum length of particulate rods and needles to the ECD for years, and maintaining this historical database is important to them. An alternate practice is to use maximum length estimates as a conservative, worst-case stance, thereby generating counts for particles as if they were all diameters of spheres. This conservative approach raises a warning flag for formulations laden with thin crystals as "acceptable" by USP definition, when objectionable by practicality. Use the microscopical evidence for its true value, seeing particles resident and dry on the membrane, as a complement to the LO data.

Ultimately, all sterile injectable products and certain topical (ophthalmic) products must meet compendial visible and subvisible particulate guidelines. The requirements for visible extraneous particulate matter are simple and are typified by the USP description:

> **USP <788>** *"... injectable solutions ... are essentially free from particles that can be observed on visual inspection...*

The USP has stringent rules regarding Foreign Matter, "...every care...to prevent contamination with microorganisms and foreign matter...each final container be subjected individually to physical inspection (whenever the nature of the container permits)...every container whose contents show evidence of contamination with visible foreign material be rejected" (33); however, USP makes no attempt to describe inspection conditions. Both Japanese (34) and European (35) guidance are more explicit in regard to light intensity, type, and method. The guidelines apply to reconstituted solutions as well.

Dry solids for reconstitution are constituted at the time of use and must comply for:

Completeness and clarity of solution

a. The solid dissolves completely, leaving no visible residue as undissolved matter.
b. The constituted solution is not significantly less clear than an equal volume of the diluent or of Purified Water contained in a similar vessel and examined similarly.

Particulate matter constitute the solution as directed: the solution is essentially free from particles of foreign matter that can be observed on visual inspection.

Therefore, for all forms of parenteral products, in a development stability matrix, parameters we would routinely evaluate in sequence include

a. color of cake (sterile powder)
b. reconstitution time (sterile powder)
c. color of reconstituted/all solution (all)
d. clarity of solution (all)
e. particulate matter evident by inspection (all)
f. pH (all)

Particle Quantitation Methods

Coulter Counter Electrical Sensing Zone Method, Size Range 0.4 1200 μm

The sample is diluted into a weak electrolyte solution and drawn through a small aperture, passing between active electrodes, interrupting an electric field. Response is based on the displacement volume of the electrolyte, and thus sizing is in geometric volume means. The response is unaffected by particle color, shape, composition, or refractive index. Suspending all but simple formulations in the saline buffer electrolyte, however, may cause a number of unwanted assay artifacts because of formulation instability or particle changes. Coulter is an excellent choice for dispersed powders, simple solutions, and suspensions (36).

Laser Diffraction Size Range, 0.1 600 μm

Operating in a wide size range, these high-performance instruments are most useful for dispersed systems and evaluation of bulk powders. In laser diffraction particle size analysis, a representative "ensemble" of particles passes through a beam of laser light, which scatters the incident light onto a Fourier lens that focuses the light onto a detector array. With specific algorithm, particle size distribution is inferred from the collected diffracted light data.

Photon Correlation Spectroscopy, Dynamic Light Scattering, Static Light Scattering Size Range <1 nm to 6 μm

Particle size is determined utilizing fundamental light properties. In diffraction instruments, the angle at which the light is diffracted depends on the wavelength of the light and the particle size. The angle of diffraction is measured to determine size. For a particular particle size, should the wavelength change, the angle will change. Using light frequency, the number of waves pass through a given point per unit time. Higher frequency is recorded as more waves cross the point or as the distance between waves shortens. Frequency change or shift information is used in dynamic light scattering. These instruments are essential in probing the submicron and nanodomains of the product fluid, with formulation character and stability as primary goals (37).

Image Analysis Static: 0.2 μm to 100s of μm Range

How can one improve on optical microscopical examination of material? By retaining the realistic views of optical microscopy in the optimal illumination, digital images are captured and further deconvolved using the software systems in image analysis. Although a quite powerful technique because of operator control and manipulation of the data, it is also problematic for primary records and product release for the same reasons. One may ask, what has been done to the primary particle(s) in question? To facilitate particle study, one may select filters to screen out unwanted artifacts or particle populations (circularity, aspect ratio, etc.) and then commence study of the selected population set.

Image Analysis Dynamic: 0.5 μm to 100s of μm Range

As in the static systems above, but utilizing image capture in dynamic, flowing systems, such as undiluted product fluid. Dynamic image analysis utilizes the microscopical components

(*i*) illumination, objective lens, and focusing lens elements, and adds fluid pathway and flow cell, plus camera, and processor for acquisition and analysis. The realistic views of optical microscopy for particles in situ augment interpretation of other in-suspension measurements that cannot visualize the particles in study.

The advantage of dynamic flow microscopy is size and feature analysis of the actual particle image, not calibration against a response curve for derived properties such as equivalent circular diameter (ECD) or geometric volume mean (GVM) or similar indirect measurements.

Sterile Injections, Suspensions, Emulsions

This discussion concerns the evaluation of parenteral formulations in a candidate product development program.

Compendial Methods for Parenteral Products

Global compendial organizations provide guidance for the local use of the pharmaceutical product. This guidance includes (*i*) definitions of products and uses, (*ii*) monographs containing medication-specific criteria, (*iii*) general rules for the use of pharmaceutical products, (*iv*) guidance for manufacturing, (*v*) guidance for physical facilities, (*vi*) specific test methods, and (*vii*) reference standards.

Compendial Considerations for Pharmaceutical Parenteral Products

USP

Parenteral products are defined by the USP in General Chapter <1> *Injections* as products intended for injection through the skin or other external boundary tissue in single-dose or multiple-unit containers, with careful control of sterility, pathogens, particulate matter, and other contaminants (38). The definition is shared by other compendia and by major compendia JP and EP. The particulate matter requirements are applicable to all liquid formulations and certain emulsion, liposomal, microparticulate candidates, regardless of container type. The concepts of sterile parenteral parameters have been applied to ophthalmic formulations recently, with guidelines for particulate matter quality of ophthalmic products are similar to parenteral products (39).

Global Particulate Matter Guidelines

The impetus for harmonization of the particulate matter determination methods came from the Pharmacopeial Discussion Group (PDG). Harmonization of compatible procedures is a primary goal of PDG. All three compendia have the same version, except for national text and the slight difference in SVI definition by JP, to exclude 100 mL nominal fill.

World Health Organization

The World Health Organization (WHO) maintains the International Pharmacopeia (Ph. Int.) in a broad guidance reference that does not specifically address particulate matter in injectable products (40). The WHO philosophy considers the first-tier issues of medication identity, safety, and efficacy. Injection safety is of utmost importance, followed by the necessity of injection therapies.

Major compendia include European Pharmacopeia (Ph. Eur.) and Japan (JP). Two methods for particle numeration have been harmonized through the efforts of the Pharmacopeial Discussion Group principally for USP, EP, and JP. These are the methods described earlier, LO and MM. The related particle content limits are contained in USP Chapters <788>, Ph. Eur 2.9.19 (41), and JP 6.07 (42). Both methods are suitable for the evaluation of subvisible particles in a variety of parenteral product formulations and presentations and may be used in development of formulae for other product forms, with appropriate validation. The same methods and product limits are endorsed by all. The intent of both LO and membrane methods is to provide robust means to assess total subvisible particle content in the commercial product.

PM that may be seen visually, USP Chapter <1> and in subvisible range, USP Chapter <788> are addressed. There has been an evolution of particulate matter measurement methodology and acceptance criteria since the inception of public domain limits for the content of particulate matter in parenteral products. In 1975, with USP XIX (43), particle matter in large volume injection (>100 mL nominal fill) parenteral products was determined by membrane-based testing with particle limits of not more than (NMT) 50 particles per mL $\geq$ 10 μm and NMT 5 particles per mL $\geq$ 25 μm. Since this initial membrane microscopical method was applied for LVI products, much investigation and method revision has occurred in USP guidance. LO was developed and implemented as the preferred or primary method, largely because of method control and efficiency.

The current methods and their reliability have been continually reviewed and improved through the efforts of USP expert committees, contribution from industry, and assistance by industry specialists overseen by the USP-PPI (Parenteral Products Industry) expert committee. Certain formulations cannot be tested by either method, such as extraneous matter in sterile suspensions. The current methods are now also applied to ophthalmic solutions in the United States (44).

Compendial Considerations for Pharmaceutical Ophthalmic Products

A new U.S. guideline for ophthalmic products was official in 2004. The evaluation of ophthalmic formulations is conducted quite similarly to that of the parenteral candidate product development program. Ophthalmic products are defined by the USP as products intended for topical application on the eye; however, they have been applied for intravitreal, conjunctival, and subtenon injection for eye therapies. Careful control of sterility, pathogens, particulate matter, and other contaminants apply.

Certain ophthalmic products must meet compendial visible and subvisible particulate guidelines, as defined in USP Chapter <789>. The guideline refers to the parenteral chapter <788> for methodology as well:

> **USP <789>** *"... topical preparations ... are essentially free from particles that can be observed on visual inspection ..." "... particle content is limited ..."*

For ophthalmic products particulate matter determination, note that only per milliliter limits are used, and that if the MM method is run, an additional size threshold must be reported as shown in Table 11. Thus, visual inspection and subvisible particle load evaluation is prudent and necessary for proper development of both parenteral and ophthalmic products.

PARTICULATE MATTER CONTAMINATION

Medical Impact—Physical Blockage

The presence of particulate matter is certainly unwanted in parenteral ophthalmic products, and care must be taken to minimize the content. What content or level matters? The answer has never been simple or dogmatic, since the "insult" must be measured in regard to absolute number, at given size, mode of entry (pulmonary, venous, etc.), duration (chronic or acute?), patient health and patient resilience. These last two factors are real but do not present reasonable consideration; we do not grade products in regard to the capacity of the patient to accommodate them. Medical effects from particle "insult" include infusion phlebitis, pulmonary granulomas, pulmonary arterial lesions, severe pulmonary dysfunction, and loss of functional capillary density of post-ischemic striated muscle to death (45).

Table 11 USP Chapter <789> Subvisible Particle Limits for Ophthalmic Solutions

Method 1 LO		Method 2 microscope		
≥10 μm	≥25 μm	≥10 μm	≥25 μm	≥50 μm
50/mL	5/mL	50/mL	5/mL	2/mL

Pathophysiologic Mechanisms

The obvious and direct effect of undissolved solids in the injected liquid is mechanical blockage of small caliber arterioles. Other effects include activation of platelets and neutrophils, generation of occlusive microthrombi (46).

Why has there been so much work and concern regarding particulate matter in the pharmaceutical industry and regulatory agencies? Intuitively, one may conclude that injecting solid extraneous matter into human arteries is an objectionable practice. Indeed it is. Certainly the foremost concern for the population receiving the injectable product is sterility, and extraneous matter is a known vector for the transmission of microbial contamination. Secondarily, minimizing the content of foreign matter delivered to the patient is certainly important. Finally, counting and tracking the content of extraneous particulate matter is an important process control tool. The "Holy Grail" of particulate matter control is a product with zero extraneous matter content. Are there known levels of particulate matter that cause physiological problems?

Among the many reports that detail the harmful effects of extraneous solids injected into the human arterial system, all agree that the content of particulate matter must be minimal and constantly sought to be minimized. In comprehensive reviews of pharmaceutical product quality, the evidence of human systemic contamination and physiological damage in high-contamination doses is obvious. Injected particulate matter is medically objectionable because of the potential for capillary emboli, for example, sudden obstruction of a blood vessel by debris. Recently, Carpenter et al. (47) proposed that subvisible particles below the current compendial limit (<10 μm) promote protein aggregation and must be monitored and controlled in therapeutic protein products. Just where do agree the line in the sand should be? I'm not sure we will ever have a single load and size limit.

The concern does not end at the therapeutic agent alone; product use and combination are also issues. For example, multicomponent admixtures aseptically compounded from multiple source containers that individually pass particle size limits of <788> may collectively contribute a large particle load. Evidence of this effect has been shown with physical confirmation of glass fragments and cotton fibers in the pulmonary arterial system during the *post mortem* examination of infants receiving parenteral therapy (PN) (48).

Thus, the primary medical risk from elevated particle content and patient insult is capillary embolism, causing interruption of blood flow that provides oxygen and nutrients to cells (49).

Utilizing these methods is an important measure for reducing these risks to the ultimate consumer, the ill patient. While it is doubtful any commercial product presents an extreme level of risk, we assay and track the particle level because particulate matter may present a physiological problem for the patient, especially when administered to certain patient populations at high levels and over time. The fundamental reasons to measure particle load content and evaluate process trends provide the means to understand control and improve product quality. Indeed, researchers have argued that pursuit of reduced contamination is less one of physiological impact to the patient, but more an indicator of improved process quality control (50).

STABILITY—DESIGNING ROBUST FORMULATIONS

We mentioned that investigation and optimization of the formulation and the subsequent production process occur during development. The particle determination methods are key to this development. Table 12 shows the categorical types of particles that will form significant particle species.

Both compendial particle counting methods tabulate solid matter and may be skewed by certain artifacts. While it is the intent of these guideline methods to measure the content of low levels of extraneous matter, particulates arising from other phenomena and product component interactions will also be detected. Even a low level content of extraneous matter at time of release may be from a single-event addition, a point source. Also present may be intrinsic-sourced material, which must be rigorously detected, examined, and removed during

Table 12 Varieties of Particles Within Two General Categories of Source

Extrinsic external	Intrinsic internal
Natural	Product package interaction
Vegetative	Hydrolysis
Anthropogenic	Leachates
	Corrosion
Manufacturing	Active ingredient/component change:
Metals	Degradation
Polymers	Hydrolysis
Corrosion products contributed	Salt forms
Extracts from points of contact	Oligomer forms
Cleaning processes	Nucleation/crystallization
Filling arena	Coalescence
Water	Sedimentation
Materials	
Air quality	
Personnel	
Equipment	
Package cleanliness	Sedimentation
	Impurity content and growth
	Process contributed
	Extracts/leachables, primarily from package

evolution of the product form. These particle sources may represent significant formulation instability and may result from one or more of the following:

- Process control failure,
- Poor formulation design in regard to use, storage, compatibility,
- Special concerns of biomolecule formulation stability, such as protein aggregation
- Adverse interaction between the formulation and the container/closure system,
- A package system that is archaic or unsuitable for the fill
- Leaking or excess vapor loss,
- Uncontrolled or unknown excipient quality,
- Active ingredient quality.

Robust and stable formulations do not occur by chance. Utilizing a comprehensive development program of formulation, physical and chemical stability evaluation is a prudent step in the product development process, and also yields assessment of the product appearance and measurement of particulate matter content. Development stability programs must include USP <1> product appearance (package aesthetics and verification of particle-free final package) and USP <788/789> particulate matter content, in statistically relevant sampling schemes that probe the batch population and indicate the appropriate sampling levels for the product process. These measures conducted during development help ensure that commercial product quality will remain on track through shelf life.

While it is not the purpose of this program to train you in chapter <1> guidelines, one must appreciate the connection between chapter <1> Injections, Foreign and Particulate Matter, the particulate matter determination described in <788>, and accordingly, <789>. Analytical approaches and sensitivity and manufacturing controls and equipment continue to be refined, and the improved physical and chemical quality in modern pharmaceutical products has been remarkable. Simple product appearance methods and more sensitive visual inspection methods must be appreciated for their ability to detect low levels of heterogeneous, insoluble, nonvolatile substances not detected by instrumental means. Beginning in early development, the use of visual inspection and human evaluation of product stability sets and a variety of illumination configurations must not be underestimated. Collimated light beam

inspection of the liquid formulae reveals light-scattering phenomena or Tyndall from submicron particle populations. More quantitative techniques such as nephelometry, turbidimetry, and color determinations provide early detection of physical change and instability in product forms.

But where does the boundary between visible and subvisible particle size (and type) exist? One cannot utilize visual inspection alone for the larger particles, just as assays for subvisible particle content also reveal particles in the near-visible sizes. Fundamental to the development of robust formulae in stable packages is utilization of many methods of overlapping detection to reveal all variations of contamination. Most of the R&D methods do not carry through to commercial target release assays, having established the fundamental properties of the product and direction for improvement in early phase operations.

In this approach, R&D and production understand each others' goals and needs and work in concert to attain robust final product within the design of production systems. Rigorous investigation of API, excipients, vehicle, container/closure, their process streams, and compatibility are essential in effects such as color, haze, precipitation, aggregation, crystal formation. The integration of visual inspection and particulate matter level determination on stability provide good sensitivity for the detection of physical changes, such as color, haze, precipitation, aggregation, crystal formation, and resultant particulate matter increase. In this design, any appearance change or particle content increase because of one or more of these factors allows their detection, isolation, identification, and data used to improve the formulation-package design.

Discussion of Particle Origin and Nature

Particles will always be present, and therefore it is the effects on the recipient and the indication of formulation integrity that are most important, in that order. With pharmaceutical cleaning, fill arena preparation and maintenance, and solution filtration to 0.22 μm nominal porosity, the inclusion of significant particle numbers is much reduced. Even single organisms included in the fill could bloom to a large number or pose pyrogenicity concerns.

Point Source

Limiting the discussion to number and size of particles is insufficient. Certainly the goal of pharmaceutical processing is a product that is consistent, efficacious, low impurity, high potency, sterile, low particle-load fill volumes that remain that way for their shelf life. But should we deem formulation meeting the public limits, or even fractional target limits, sufficient? In certain situations, even low numbers of particles may be objectionable, if from toxic sources, from known adulterants, and even from a singular or point sources. Toxicity and adulteration events are obvious; however, consideration of point sources is important. Consider the following case history:

> A batch of 87,000 ampoules were filled over a one day sequence with in line processing for washing, drying, and sterilizing the open ampoules just prior to filling. In process mil std. inspection and terminal 100% inspections showed <1% rejection for all defects, with much <0.5% rejected due to particles. Assay for subvisible particle load by LO of 10 unit pools from each of the 10 sublots resulted in no individual units and no pool average 100 particles ≥ 10 μm and 16 particles ≥ 25 μm. The lot was released, but prior to labeling and shipment, samples of the released ampoules were used for microscopical assay training exercises. The resultant counts by MM were well correlated to LO (no silicones in an ampoule to skew the LO count) at 76 particles ≥ 10 μm and 23 particles ≥ 25 μm. However, the microscopist counting the samples asked why all the particles were the same and so shiny? Examination of the particle isolate membranes by lab supervision revealed a nearly singular occurrence of one particle type, metallic flaky solids. Upon further investigation, identification of the particles confirmed them to be stainless 316L and tracked to a particular (new) valving system being used on the filler (without in line fill filter). The lot was retrieved from the distribution centers and rejected.

Why was the lot quarantined and rejected? All tests met particle specifications, for example, particulate matter content was far below the SVI products limit of 6000 particles ≥ 10 μm and 600 particles ≥ 25 μm limits per container set by USP <788> for analysis by LO

and the SVI limit of 3000 particles $\geq$ 10 μm and 300 particles $\geq$ 25 μm limits set by USP <788> for analysis by MM. Rejection was selected because the particle load was uncontrolled, difficult to observe by the 100% release inspection (too small and settled quickly) and so reinspection by any method was unlikely to remove isolated metallic contamination, and most objectionable as a particle type. The event was isolated, since this was the first use of this type of valving system. The finding necessitated the valve removal from any future processing step, launched an engineering investigation into the effects of different valve metal components, and thus isolated this incident from related manufacturing.

PROCESS CONTROL—MEASURE OF INDUSTRIAL COMPETENCE

Manufacturing Arena

Federal Air Standards

No discussion of particulate matter in parenteral medications is complete without reference to the expectation for air quality in the assembly and filling zones curtaining our package preparation, setup, filling, and capping. The air quality and personnel gowning and movement within these zones has significant impact on the sub-10 μm particulate content in the final product. Historically guided by federal standard 209E and currently by corollary ISO 14644 classification, ISO class 5 is equivalent to historical Federal Std. 209E class 100 (51). Filling arenas operating at this level will sample three locations, and minimal 19.6 L volume and require the following airborne particulate matter level limits:

Count	Size (μm)
100,000	0.1
23,700	0.2
10,200	0.3
3,520	0.5
832	1
29	5

While not directly contributing to the >10 μm population, the sub-5 μm population is a direct indicator of the most transmissive and contamination-bearing particle population. Spikes in the sub-5 μm population are most certainly a warning that larger particles may also be increasingly present.

Area Control

We have been discussing minimization of particles in regard to extraneous matter. Elevated levels, extraneous types, and variable content of particulate matter are all significant indications of preparation and assembly processes that are not in control. We must appreciate the fundamental requirements for pharmaceutical products in regard to safety, sterility, stability, efficacy, and purity as the essential foundation of commercial pharmaceutical products and often relating to the assembly process. The selection of raw materials, storage, assembly, and packaging of the final product must be derived from the development process and well controlled for commercial production. Modern pharmaceutical filling arenas are designed such that visibly sized, environmentally related particles are minimized in the final package, since equipment and processes have been configured to minimize exposure of the product fill and package interior to extraneous matter. Form-fill-seal processes are good examples of the utmost commercial filling design refinement. Process streams starting with the vendor are often configured specifically for the product, and at a minimum, components are selected and optimized on the basis of the properties of the formulation.

At the end of the process, normally prior to labeling, 100% final package inspection is conducted to reveal the presence of any visible particulate matter that may have been included in the product. Inspection systems and their reliability are important because of the relatively insensitive detection at particle sizes approaching the lower detection limit (which we stated as 100 μm) and the upper end of the detectable size ranges by the primary method, LO. Although

visual inspection is based on human detection capability, many pharmaceutical firms utilize machine-vision inspection systems calibrated as equivalent to, or more sensitive than, the human inspection methods.

Visibility of particulate matter is a function of several parameters but can be grouped in three categories: (*i*) operator acuity/machine sensitivity, (*ii*) particle physical properties, and (*iii*) inspection conditions. Many companies use a combination of (*i*) machine and human systems, (*ii*) serial inspections, and (*iii*) trained quality control auditing to increase the reliability of detection.

While product batch acceptance is dependent on subvisible particle content determination by these USP <788> methods, most new drug applications (NDAs) are filed with LO test data that also provide an excellent means after market launch to survey the batch quality through count levels and trending, minimizing the risk of defective packages from entering the marketplace (24,52).

In addition to verifying the batch meets compendial expectations, quantitation of particulate matter by either method provides a means to study process variations that may affect the product. Full understanding of the particle population by visual inspection alone is not realistic because of the limits of visual resolution (i.e., variability in visual acuity between persons and observations by the same person, as well as failure to observe specimens appropriately and the probabilistic nature of detection. Quantitation of particles is essential for trending and setting realistic (and improving) internal limits for batch quality, which will vary somewhat on the basis of the type of parenteral or ophthalmic formula and the packaging system. Membrane testing for quantitation is also an appropriate prelude to more extensive methods for the characterization and identification of particulate matter. Not only will the particle types isolated indicate potential stability and assembly issues, even small populations of single-type contaminants indicate a point source of particulates to be eliminated. Insight to the identity and thus cause(s) of elevated particulate matter will facilitate their control and minimization (53).

Nath et al. (54) has written a stimulus to the revision process to comment on the level of particulate matter in Office of Generic Drugs review of 295 ANDAs. Included were liquids and lyophilized and powder forms of parenterals, filed from 1998 to 2002, covering 51 firms and 110 drug substances. The data was determined primarily by LO. In these files, batches contain significantly less particle load than the current compendial limits, and the paper position is that compendial limits are far too high. Trends in mean counts were evident for aseptic versus terminal sterilization in package forms and in product forms. Nath and his coworkers detailed the "wide gap" between the current USP limits and the performance of freshly filled drug products. Mean counts for particles $\geq$10 μm per container were 27× and 40× lower than USP limits of 6000 $\geq$10 μm and 600 $\geq$25 μm, respectively.

Pharmaceutical and Delivery Systems

Devices and Delivery (IV) Sets

To maintain the integrity of the product, we have promoted the concept that one must carefully design and control the emerging formulation design with a carefully selected package to ensure continued stability. This is a difficult and complex task in pharmaceutical development, and especially so with shortened time frames, increasing use of innovative devices, which may be developed without good appreciation for the effects of contamination sources, and increasing use of non-core company resources of material and supply, which expands the "universe" the developer must understand and control.

The key element of the new formulation may be a one-of-a-kind device or process that is being licensed to good market advantage. Does the formulator understand the mechanistic design, the supply and control of components, and the process? Has it changed recently. . ..will it change without much control? If essential for the product and proprietary, can the formulator review for the innovator/supplier the concerns regarding particle addition and formation pathways, so as to prevent them? Are there details of the industry known to the practitioner, but not apparent to the user that may present a PM challenge? Sorting out answers to these questions early in development is advised, and use of stability set review is essential to predict final product stability and use issues.

PARTICLE ORIGINS

Additive/Extrinsic

Extrinsic particle types originate by *direct contamination or addition*. These are often singular events, although may occur repetitively and with multiple particles per event. The key is that extrinsic particles are often an event and are unchanging; the added particle(s) will not generally change over time unless there is fragmentation into smaller bits because of mechanical agitation or chemical activity (corrosion). PM typical of extrinsic origin will include paper, fibers, geological matter (soil minerals), biological matter (vegetative, insect), and airborne particles <10 μm.

The types of particles in extrinsic contamination events are environmental, machine-related (unless representing product effect on the machine), personnel-borne, as a result of inadequate preparation or cleaning.

Multiple Event/Multiuse Package

In certain cases, the extrinsic single event reoccurs because of reuse of the product, especially in regard to the closure integrity to resist coring (single large particle) and to resist shredding or otherwise losing filler material so common in elastomers. The source of particulate matter is finite; however, very little is needed to cause a defect.

Growth/Intrinsic/Changing

In considering the entire pharmaceutical package, the level of particulate matter that may be seen or may cause excessive particle load or even failure to meet compendial subvisible count limits is exceedingly low. Consider the example of particle number versus concentration in Table 13.

In the following examples, consider from the above table just how little content or change may promote an undesired particle event.

Package Change

Leaks and evaporative events may initiate change for actives that readily form crystalline hydrates. As the formulation loses water vapor, the elevated potency may be detected through stability assay and addressed. If the change is occurring for only a few containers or not sampled, the vapor loss may result in the nucleation and crystallization of the API to a less-soluble hydrate form, inducing a cascade of many more crystals. Package defects and other (extrinsic) particles present in the fill volume would serve as potential nucleation sites for this event.

Ingredient or Active Purity/Change

Particles will form as chemical changes in the active ingredient or formulation excipient produce less-soluble moieties over time. Any insoluble forms >0.2 μm are removed by sterilization filtration and the remainder <0.2 μm if unchanging, remain as trace levels of

Table 13 Relationship of Particle Count, Size, and Concentration for Selected Cases in a 10 mL Fill Volume

Particle count	Particle size	Particle concentration[a]
10,000	2 μm	0.004 ppm. . .(subvisible, not reported)
10,000	10 μm	0.52 ppm. . . . . (subvisible, at threshold)
1000	25 μm	0.82 ppm. . . . . subvisible, but visible if viewed after settling, then swirling container
1	100 μm	0.05 ppm. . . . . just visible
1000	2 μm equant	8 ppb. . . . . visible due to light scatter
Subvisible		
100	5 μm particles = 0.125 μg mass = 12 ppb in 1 mL	
50	10 μm particles = 0.5 μg mass = 50 ppb in 1 mL	
5	25 μm particles = 0.781 μg mass = 41 ppb in 1 mL	

[a]Density = 1 g/cm^3.

formulation impurities. These forms may appear (in chemical assays, and as subvisible particles, and even to visible sizes at higher concentrations) as their concentration builds above their solubility product in the formulation, in storage, over the shelf life of the medication. Very little material is needed to yield a single, visible particle, or significant amounts of subvisible particles >10μm to yield elevated PM assay counts (Table 13).

Product Package Interaction

The integrity of the formulation has much to do with the lack of product-package interaction. We desire an inert package with the product fill. Ions will seek equilibration in the product fill from all product contact surfaces and dissolved ions from the formulation constitute the new formulation "stew" from which new more insoluble particle may form. Drug ingredient with low solubility product (K_{sp}) for any available ion will readily form solids, and often well-formed crystals, especially at promoting temperatures during storage.

Glass corrosion occurs in borosilicate tubing vials on exposure to base and elevated temperature. The effect is 25 times more severe in mild base around pH 8 than in mild acid around pH 4 (55). The inner glass wall corrosion is pronounced at sites of extreme container formation temperatures, such as the base. Glass borosilicate ampoules with much thinner walls have not shown the same effects, because of the much lower level of finishing heat needed to form the container. Similar corrosive effects have also been promoted by so-called aggressive solutions, such as high concentration solutions with counter ion, such as sodium bicarbonate and drug phosphates. Sulfate-containing formulations have shown white ring deposits on the inner glass sidewall, at areas of greatest heat influx, and correlated barium ion migration, forming highly insoluble Ba_2SO_4 crystals on the glass reaction surface (56).

The much more reactive and rich formulae of closures can have a significant impact on the formulation as material physically falls from the elastomer and extractables exchange with the formulation fluid. As in the above examples, the chemical equilibrium drives the inclusion of small amounts of elastomer formula components in the fill, especially the volatile, low melting, small mass, and high vapor pressure moieties. If the substances have limited solubility in the fill, solid PM will form and grow and may reach detectable size ranges.

CHANGE MECHANISMS

Coalescence/Aggregation

Direct

The affinity of separate <50 μm solids to cluster group in fragile aggregates will be the fundamental cause of size change for microparticulate matter. The adhesion of particulate matter, whether homogeneous or heterogeneous, is driven by weak yet effective forces (57):

1. van der Waals forces between particles
2. Capillary force of hydrophobic liquid between particles in hydrophilic matrix
3. Electrostatic forces for particles >5 μm may only be a concern in airborne and dry surface situations, as in delivery of the foreign matter to the pharmaceutical container.
4. Adsorption at point of contact, promoted by elevated temperature and time, resulting in chemisorption between particles and stronger adhesion.

The consideration of these forces is most important for preparation and cleaning of the pharmaceutical package, since in the fill solution, much of the weak force is overcome by the aqueous or oil solution.

Aided

Oils, liquids, polarity groups (friends) within matrices of opposite polarity all serve to enhance the grouping of separate solids. Silicone oil is one of the most common coalescence aids in aqueous media due to its ubiquitous use, relative insolubility, and stability. Water in hydrophobic media would have a similar grouping or adhesive effect.

Sedimentation

The smaller the particles, the less likely one will see them visually and count them analytically. In certain instances, one may visualize settled particles that were never observed when dispersed throughout the fill. Thus, it is prudent to allow parenteral fill solution to settle for a day prior to first visual examination. Centrifugation may hasten the process. Examination on a gentle swirl of the container resuspends particles and is preferable to vigorous inversion mixing and mechanical axial rotation to cavitate the fill. Slow axial rotation (a swirl by hand, maybe?) will reveal even the most light dusting of very small particles, especially if assisted by a strong light beam to impart a Tyndall effect.

Degradation

Active ingredient and to a much lesser extent due to concentration excipient components will degrade. Many factors can augment the process and may yield sufficient less-soluble product to yield particulate matter. Hydrolysis and oxidative processes are most likely; however, the change imparted by physical effects of cold, heat, shear, and light cannot be ignored in the change event.

Precipitation

This phenomenon is most probable for formulations near or above the solubility limits of the formulation components, especially the active ingredient. Ideal conditions are those with no nucleation site potential (PM and package defects), at target pH and held at insert temperature; however, changes in the formulation may promote active ingredient precipitation. The most common and likely event is for active ingredient with significant insolubility for certain common counterions, such as cations Al, Mg, Ca, K, Ba, and all transition metals. Ionic extracts from the package must be considered, and while bulk content is important, surface concentration for all formulation contact is very significant. For inorganic substances with very low solubility products, storage contact with formulations containing simple buffering systems of sulfate, phosphate, acetate, and even higher mw counter anions may promote precipitation to yield elevated particle count in a wide size range. Precipitation of barium sulfate crystals was evident on the glass surface of a container with available (extractable) Ba^{2+} in contact with the high concentration of drug anion and bisulfite ion of the antioxidant (58,59). The aforementioned example was from a glass package with BaO content, but at a minor constituent concentration of 2%.

Closure systems were common sources of extracted trace ions promoting particle formation with actives and buffering systems until reduced and alleviated in some cases by improvements in elastomeric formulation to remove highly mobile and components with high counterion capacity. Barrier systems of Teflon and proprietary polymer systems on the formulation face of the closure have been very successful.

Nucleation

Consider the minute sites responsible for the promotion of growth of a singular species to be a nucleation event. In our consideration, the heterogeneous nucleation phenomenon is most important; for example, a foreign body nucleating the growth of drug crystals from solution. Certainly, any material may provide a sufficient platform; however, small inert solids such as glass or minerals are ideal nucleators. Defect zones consisting of rough surfaces on the package walls also provide good sites for particle formation. One can also consider silicone oil or immiscible liquids in the pharmaceutical fill to be potential nucleators, although their promotion of crystal growth is much less likely than as formers or gatherers of material.

Crystallization

One of the most dramatic events one may encounter in formulation stability is the generation of crystalline solids, significant as stability failure and in a dynamic process, crystal generation indicates a serious challenge for the formulation. PM may be present or form from API impurities and degradation products. Larger (visible) PM may be present even from ppm concentrations. Most critical factors are relatively insolubility in the product solution -pH, ion content, temperature, and dilution. In situ salt formation is sometimes performed during

manufacturing process, acid or basic impurities dissolve in the product during the titration process even after the pH is uniform throughout the solution, those dissolved impurities could still exist in solution in a supersaturated state, not removed by filtration. The dissolved impurities precipitate over time, leading to product failure. API may form a gel after dissolving in solution and take a long time to solidify, especially at low concentrations of API. The gelling process typically is preceded by haze formation, with detection aided by Tyndall beam light. Finally, incompatibility between the active ingredient and diluents used in admixing and preparation can be another source for PM in the final dosing solution.

Nucleation
Insoluble impurities may be present in a more soluble form (amorphous) and precipitate in final product in a more stable form (crystalline).

Hydrate or Solvate Formation
These forms are more thermodynamically stable than the parent molecule and often less soluble than the anhydrous form, thus constitute significant threat to formulation stability in high concentration formulations experiencing dehydration, and volume loss. Accordingly, solvate crystal forms may also occur, though rarely in aqueous formulation. Hydration of proteins is essential, often occurring at high percentage of the drug volume and so hydration effects are more pronounced in these formualtions.

Polymorphism
If conditions allow, especially in high concentration formulations, alternate physical structures may nucleate and reside in solid form. Chemically identical, however, different physically, the formed polymorph is the end of the stable formulation as intended, since return to the original polymorph is quite unlikely energetically. Again, the key for remediation of any crystallization event is full understanding of the solubility and stability parameters for the form and any causative events, such nucleation seeds or surfaces, pH change.

Salt Formation
Much more likely than solvates and polymorphs, newly formed, less soluble salt forms are a danger in packages with potential for ion exchange, such as (*i*) sealing with elastomeric septae, (*ii*) formulation components that may contribute ionic character, (*iii*) pharmaceutical water, (*iv*) insufficient cleaning of components, and (*v*) ionic species from the glass container.

Impurities
The presence of trace to low concentration impurities is common and more pronounced upon storage. Formulation physical stability may be compromised by the growth of impurity-related particles, especially for relatively insoluble moieties, in the presence of foreign material, immiscible liquids, and a growing population of other impurities, soluble or insoluble.

Micellar Change
Many drug moieties are amphophilic, for example, drug molecules with a polar (hydrophilic) and nonpolar (hydrophobic) characteristics, primarily due to functional group characteristics. This is characteristic of detergent molecules, and hence their desired capability to "bridge" grease in water, and of many pharmaceutical compounds as well. The hydrophilic and hydrophobic ends of the molecule align in solution, forming micelles, or associations of molecular polarity with nonpolar cores in the aqueous formulation. Certainly, more complex variations of the micelle in nonaqueous systems and in conjunction with selected hydrophobic materials allow micelles to "carry" the relatively water-insoluble substances in an aqueous medium. The associated groups are small in number (up to 100 molecules is typical) and in size, ranging up to 10 nm. The micellar group can solubilize other hydrophobic substances, as a function of surfactant concentration. There is a critical concentration above which the

surfactant enters the micelle and the monomeric surfactant concentration remains constant. This is called critical micelle concentration (CMC). This phenomenon is particularly evident when excipients held in the micelle are subsequently released upon dilution of the formulation, brought about by dilution of the surfactant concentration below the CMC.

Micellar formulation stability depends on the stability of the small micellar groups. Imagine the effect on the molecular group where another hydrophobic material has been entrained in the hydrophobic core or is competing with the parent molecule. This competition is most significant in the cases of extracted organic moieties, such as plasticizers, for drug analogs, impurities, and degradation products that are more hydrophobic than the parent and alter the solubilizing activity of the parent micellar groups. The importance of the micellar activity in particle formation or, more realistically, particle appearance, is that seemingly stable, clear formulations can suddenly appear cloudy with the onset of nucleation, aggregation of material previously held in solution, and now falling out of the micellar groups due to competition with more amenable substances.

Oligomers

Monomers or single molecules will join through chemical processes to form dimers, trimers, and oligomers (a limited assemblage of monomers, short of polymerization). The importance of the larger molecules to the solution integrity is their inherent solubility, especially in regard to micellar active ingredient formulations and with specific emphasis for subsequent dilution of the formulation. How can a longer chain of the drug or even drug analog be a problem as particulate matter? It is directly related to solubility, and thus most prevalent in high concentration and saturated formulations, and those requiring micellar association for stability (solubility).

Leaching/Extraction

Extractables: "Substances that can be extracted from plastic materials/systems using extraction solvents and/or extraction conditions that are expected to be more aggressive than the conditions of contact between the material/system and a finished drug product."

Leachables: "Substances that are present in the final drug product because of its interaction with a plastic material or system (60)."

PM may not be evident until well into the shelf life of the drug product due to slow changes in the formulation and trace levels of leached substances. At each point of contact for components of the formulation, potential for including extracted substances must be investigated. A case history describing the bloom of an extracted substance in a concentrated active formulation occurred because of the extraction of a phthalate ester from a process filter-housing into the active moiety mother liquor during synthetic process yielded a significant visual cloud in the otherwise acceptable ampoule injectable product (61).

Particle Detection—Inspection

One must appreciate the connection between USP guidelines for packages, visual guidelines, and subvisible testing. Analytical approaches and sensitivity and manufacturing controls and equipment continue to be refined, and the improved physical and chemical quality in modern pharmaceutical products has been remarkable. Simple product appearance methods and more sensitive visual inspection methods must be appreciated for their ability to detect low levels of heterogeneous, insoluble, nonvolatile substances not detected by instrumental means. Beginning in early development, the use of visual inspection and human evaluation of product stability sets and a variety of illumination configurations must not be underestimated. Collimated light beam inspection of the liquid formulae reveals light-scattering phenomena or Tyndall from submicron particle populations. More quantitative techniques such as nephelometry, turbidimetry, and color determinations provide early detection of physical change and instability in product forms.

But where does the boundary between visible and subvisible particle size (and type) exist? One cannot utilize visual inspection alone for the larger particles, just as assays for subvisible particle content also reveal particles in the near-visible sizes. Fundamental to the

development of robust formulae in stable packages is utilization of many methods of overlapping detection to reveal all variations of contamination. Most of the R&D methods do not carry through to commercial Target release assays, having established the fundamental properties of the product and direction for improvement in early phase operations.

In this approach, R&D and production understand each others' goals and needs and work in concert to attain robust final product within the design of production systems. Rigorous investigation of API, excipients, vehicle, container/closure, their process streams, and compatibility are essential. The integration of visual inspection and particulate matter level determination on stability provide good sensitivity for the detection of physical changes, such as color, haze, precipitation, aggregation, crystal formation, and resultant particulate matter increase. In this design, any appearance change or particle content increase because of one or more of these factors allows their detection, isolation, identification and data used to improve the formulation-package design.

Light Obscuration Vs. Microscopy

The preference for the LO analytical method resides in its general availability, common use, and experience among the manufacturers. Instrumentation, calibration schemes, and vendor support make this method a far more routine and regimented application. The modular aspects of the instrument with strong vendor support, especially in calibration, make it the logical first-pass for particulate matter determination. The negative aspects of LO include the counting of artifacts (oils, air) and a volume-based sizing that often renders a low-bias size determination. Indeed, there are many parameters of LO operation one must control, which are shown in Table 14 (62).

MM has high value in revealing the nature of the retained particles and the type; however, it requires much more specialized training and may be considered imprecise without careful lab control. Membrane methodology is time consuming due to its labor-intensive nature and has several operational parameters that must be controlled or will add to counting

Table 14 Light Obscuration Assay Defects

Inherent (instrumental)	Applications
Size error:	**Calibration error:**
Pulse height ~ projected area (sphere is best) Particle nature Under sizing of transparent vs. opaque/color	All calibrations are secondary because they do not match the particle size parameter they are used to measure; e.g., how many polystyrene latex beads are in your solution?
Between system *variation*: sensor type	**Coincidence effects** *Undercounting* hidden particles *Oversizing* two or more smaller particles counted as larger particle
Optical considerations: ↑ Refractive index difference ~ ↑ size	Artifacts: • Air bubbles counted, • Release of dissolved air (negative pressure sampling), • Immiscible oils light affected by subpopulation, shift of baseline (added pulses) • Incompletely dissolved drug
Flow too slow: ↓ S/N	Sampling variability Stratification of natural particle types Small sips in large volumes LO sample relative to batch Inadequate mixing
Focused sampling: sips of 5 mL but representing what volume?	Counting efficiency Serious undercounting above 25 μm
Sample bias: large, dense particles settle	Sample volume LO samples 4 × 5 mL volumes If the sample pool is large (250 1000 mL) the coefficient of variation will be much larger than that from a 100 mL pool

Source: From Ref. 62.

Table 15 Improved Microscopical Assay Membrane Microscopy Assay Defects

Inherent (instrumental)	Applications
Artifacts: membrane defects and preparation damage	Sizing: Graticule or linear scale used? Determining ECD by "mental" comparison, or by max. chord
Illumination: may be too low and/or misaligned Magnification : 100× ± 10×? USP graticule ± 2%?	Counting: Manual tabulation errors Blank: low and controlled enough?

Source: From Ref. 63.

and sizing error (Table 15) (63). Both tests are valuable and practitioner labs must have proficiency in each method. Key to these methods is the concept that there is no single ideal method, and development units must be adept at all compendial and alternate methods of assay (64). Strong formulation development and new product testing laboratories will maintain both methods.

PARTICLE IDENTIFICATION

Introduction

To control and eliminate particulate matter in the final product, the source must be controlled or removed from the assembly system. The ideal is to identify each particle or at least categorize in pursuit of the source. The manner in which the identification is pursued follows a stepwise and logical path, mediated by microscopical methods. Why microscopy? Advantages are that it (*i*) gives us the direct view into specimen state, (*ii*) requires minimal sample ng-mg, (*iii*) instant recognition for many materials, (*iv*) context is evident, (*v*) associated and extraneous materials are identified, (*vi*) allows one to make the best judgment about next steps as a triage function. Disadvantages of this application are that it (*i*) requires extensive training and (*ii*) is quite labor-intensive.

Stepwise Process

A problem is usually manifested by incidence of visible particles and/or elevated subvisible particle content. The first of many steps begins with confirmation of the incident and data-mining with the observer. Clues to the identity and changes associated with the incident may reside with the initiator or owner of the process, assay, or system that discovered the particles. Representative reject containers are evaluated and confirmed present in the rejected units. Typical categories of particles by visual appearance or microscopical appearance and the nature of these isolates explored for fundamental properties and clues to their presence. In this first sequence, visual examination and low magnification stereomicroscopical evaluation aids categorization of the particles into size, physical features, solubility, hardness, reaction to chemical reagent groups. The properties are further determined to an identification using low to high magnification polarized light microscopy (PLM), wherein, refractive index, birefringence, extinction, pleochroism, crystal system, association (anything else there?), condition (what's happened to the particles?), crystallinity, more physical/chemical properties that may be conducted at the microlevel to reveal surface character, size, shape, degree of transparency, dispersion of refractive index, confirm functional groups, predict solubility (helpful for standard assays chromatography, mass spectrometry, NMR). Microfurnaces or hot stages may be utilized with PLM to determine thermal effects, showing dehydration, melt, glass transition, crystallization, nucleation, crystal transition. Obvious particulate species may be identified by this stage common to the microscopist, and often unknown by conventional laboratory assay, such as cellulosics, all forms or biological solids, fibers, hairs, insect-related matter, for example, commonly extrinsic materials. Metals, polymers, paint, glass, rubber, oils, inks may all be categorized but not identified by this time. More importantly for long-term stability, what forms of intrinsic species are present? Drug and formulation component species may be identified by crystallographic properties alone. Others may require preparation

mediated by microscopy and analysis by infrared microspectroscopy, or (Fourier transform or Dispersive) Raman microscopy, bulk X ray, examination by scanning electron microscopy with energy-dispersive X-ray spectrometry, and mass spectrometry (63).

Particle Evaluation

At this point in the analysis you may know the types of particles involved, as contamination, process equipment, formulation, package, or true active ingredient change. Is the event due to single or multiple incidents? Often an inspection method may alert the lab to the largest or most distinctive particles, but not the true cause of instability. Careful investigation of the subvisible content often reveal those species responsible for the visible event. Are the particles representative of a chemical or physical change? Are they extrinsic or intrinsic? What level is occurring, and has it changed with time? Is the origin obvious in the primary particle or indicated by secondary, low level particle load?

Particle Characterization Process Control

Minimization of particulate matter content is an important facet of the pharmaceutical assembly process. Modern filling arenas are designed such that visibly sized, environmentally related particles are rare in the final package. 100% final package inspection is conducted to reveal the presence of any visible particulate matter that may have been included in the product. Although this inspection is based on human detection capability, many companies utilize machine-vision inspection systems calibrated equivalent to or more sensitive than its human counterpart. Visibility of particulate matter is a function of several parameters but can be grouped in three categories (*i*) operator acuity/machine sensitivity, (*ii*) particle physical properties, and (*iii*) inspection conditions. Many companies use a combination of machine and human systems to detect visible particle content in the parenteral product. Requiring product batch acceptance by visual inspection and subvisible particle content by USP methods such as stage I (LO) or stage II (membrane) testing provides an excellent means to survey the batch quality and prevent defective packages from entering the marketplace (24).

In addition to verifying the batch meets compendial expectations, quantitation of particulate matter by either method provides a means to study process variations that may affect the product. Quantitation of particles by visual inspection is not realistic due to the probabilistic nature of detection. Membrane testing is an appropriate prelude to more extensive methods for the characterization and identification of particulate matter that provide insight to the cause of elevated particulate matter and thus allow its control.

Investigation of Change

Several categories of change and events are most helpful to consider in particle-generation investigations:

- Process control failure,
- Poor formulation design in regard to use, storage, component/package compatibility,
- Special concerns of biomolecule formulation stability, such as protein aggregation induced by heat, light, salts, etc.
- Inability to maintain formulation integrity because of solubility product
- Adverse interaction between the formulation and the container/closure system,
- A package system that is archaic or unsuitable for the fill
- Leaking or excess vapor loss,
- Uncontrolled or unknown excipient quality,
- Active ingredient quality

SUMMARY AND WRAP

PM content in the final package pharmaceutical product is inversely proportional to the degree of development, investigation of robustness, and the quality of commercial preparation at product assembly. Investigation and optimization of the formulation and the subsequent

production process occurs during development and is continued in the commercial arena. Particle determination methods are key to this development. Many analytical methods are utilized for particle determination, often most appropriate within specific size ranges, such as the submicron, sub-10 μm, and 10 μm to visible ranges. Both USP compendial particle counting methods tabulate solid matter but may be skewed by certain artifacts. While it is the intent of the public guideline methods to measure the content of low levels of extraneous matter, particulates arising from other phenomena and product component interactions will also be detected. Even a low level content of extraneous matter at time of release may be from a single-event addition, a point source. Also present may be intrinsic-sourced material, which must be rigorously detected, examined, and removed during evolution of the product form. More critically, what investigational methods are most pertinent to the dose form, formulation, and stability-indicating properties of the active ingredient?

In development and even for commercial testing and release, the public domain methods and relevant limits may be insufficient for full analysis and control of the final product. Deep understanding of the formulation integrity, in the selected package over time is necessary for continued high quality production. Investigation and use of additional analytical and performance methods will aid the ongoing effort. Integration of the efforts of key pharmaceutical teams in analytical, formulation, packaging, clinical, quality assurance, and regulatory will keep the products clean and stable.

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6 | Endotoxin testing

Michael E. Dawson

INTRODUCTION

Endotoxin is a potent biological response modifier, with a wide range of effects including fever, shock, and death. Endotoxin contamination of parenteral medications is of particular concern because these products are administered directly into the body, bypassing the protective barriers of the skin and the intestinal wall. Consequently, endotoxin contamination of parenteral medications must be strictly controlled to prevent deleterious effects on recipients of the products. By contrast, endotoxin contamination of topical and orally administered therapies is generally not a concern because of the effectiveness of the skin and intestine wall in preventing the entry of endotoxin into the blood and other tissues.

The bacterial endotoxins test (BET) is one of the two critical microbiological release tests common to parenteral products, the other being the sterility test. The test is imperative because of the harmful effects caused by endotoxin. The high potency of endotoxin is illustrated by a comparison with therapeutic products. The minimum endotoxin dose expected to elicit a fever response in humans in a single administration (the threshold pyrogenic dose) is five endotoxin units per kilogram body weight (5 EU/kg) (1), which is equivalent to approximately 0.5 ng/kg of *Escherichia coli* endotoxin. This is orders of magnitude less than the doses of most drug products, which typically have doses in the milligram per kilogram range. Endotoxin is clearly a very potent substance and its effects are predominantly deleterious.

Endotoxin is almost universally present in the natural environment, and it is resilient and persistent. Stringent and specific steps must be taken to ensure that parenteral products are not significantly contaminated with endotoxin, either from component materials or by introduction during the manufacturing process. Finished parenteral products must not contain quantities of endotoxin in excess of the limits specified in either compendial monographs or approved submissions. Low levels of endotoxin, below these limits, are tolerated by humans (and other species).

This chapter begins with a review of the nature of endotoxin and its role in bacterial cells. The biochemistry of endotoxin; its effects and properties; the practical implications of the properties of endotoxin for parenteral products; and standard endotoxin preparations are then discussed. The *Limulus* amebocyte lysate (LAL) reagent is introduced and the various LAL test methodologies for the detection of endotoxin are described. The regulatory context for the endotoxin testing is addressed with particular emphasis on the harmonized pharmacopeial endotoxins test chapters and the United States Food and Drug Administration (FDA) Guideline on the *Limulus* amebocyte lysate test (2). Practical information is presented on testing parenteral products and a brief section on testing medical devices is included. The chapter finishes with an overview of depyrogenation.

ENDOTOXIN

Endotoxin Structure

Endotoxin is a structural component of the cell envelope of gram-negative bacteria, which consists of an inner and an outer membrane. The inner (cytoplasmic) membrane is a typical biological membrane consisting of a phospholipid bilayer with embedded proteins and is similar in structure to the cytoplasmic membrane of gram-positive cells. The outer membrane comprises an inner phospholipid leaflet (layer) and an outer leaflet, of which the principal component is lipopolysaccharide (LPS), not phospholipid. Essentially, LPS is endotoxin. The structure of the gram-negative cell envelope is shown in Figure 1.

Because of differences in details of their structure, some LPSs have greatly reduced biological activity and toxicity. Consequently, it has been suggested that such LPSs are not endotoxins. Naturally occurring fragments of gram-negative bacterial outer membrane may include membrane proteins associated with the LPS. Others have suggested that the term

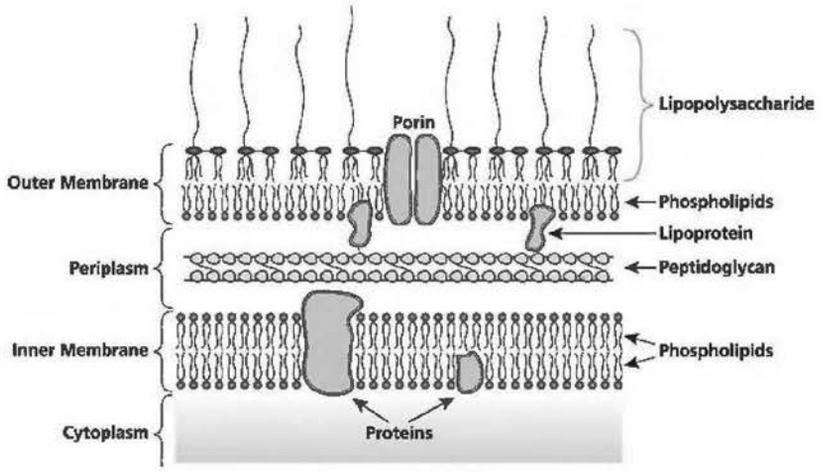

Figure 1 The cell envelope of gram negative bacteria.

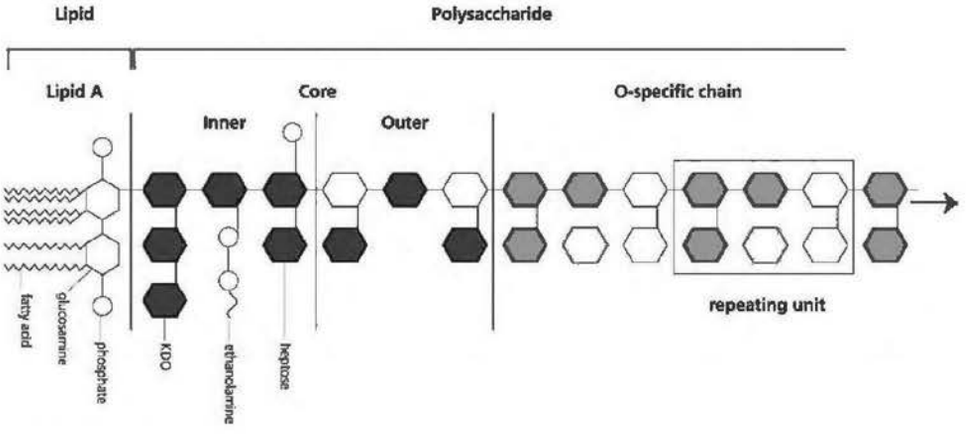

Figure 2 Schematic representation of the structure of LPS (*Salmonella typhimurium*). *Source*: From Ref. 3.

endotoxin be reserved for naturally occurring membrane fragment material and that purified material should be referred to as LPS. However, this distinction is not observed by those testing for endotoxins and the purified LPS preparations used as standards for LAL tests are generally referred to as endotoxin standards.

As the name suggests, LPS consists of a lipid and a polysaccharide moiety. More commonly, LPS is considered to consist of three regions, one lipid and two saccharide (Fig. 2). The lipid region is termed lipid A. The saccharide regions are the core and the repeating oligosaccharide. Lipid A is hydrophobic and is negatively charged. It anchors the LPS in the outer membrane. The core connects the lipid A to the repeating oligosaccharide chain, the hydrophilic, antigenic region of the structure that is presented to the environment of the bacterial cell. As a general principle, the uniformity of structure between the different groups of gram-negative bacteria decreases outward from the more conserved lipid A, to the core, to the highly variable repeating oligosaccharide.

Lipid A

Classic enterobacterial lipid A, represented by that of *E. coli*, consists of a disaccharide of glucosamine with a phosphoryl group on each of the two sugar residues. The glucosamines are substituted with fatty acid chains (generally between 10 and 28 carbons in length). The numbers of fatty acid chains are variable and may be asymmetrically or symmetrically distributed between the two glucosamine residues. The fatty acid chains are important to the toxicity of lipid A and deacylation of lipid A reduces the toxicity. Six chains in the specific asymmetric distribution found in *E. coli* gives greater activity. Similarly the presence of two phosphoryl groups is critical to LPS toxicity. Monophosphoryl lipid A (MPLA), produced by controlled acid hydrolysis of lipid A, is substantially less toxic than diphosphoryl lipid A. (Regulatory approval is being sought for MPLA as an additive, or adjuvant, to vaccines because it retains many immunostimulatory properties of LPS without the toxicity (4).)

Structure is critical to the toxicity of lipid A. The details of structure of lipid A and its toxicity vary between species of gram-negative bacteria; consequently the toxicity of lipid A varies depending on its source. Full endotoxic activity requires two phosphoryl groups and six fatty acid chains of appropriate length in the locations found in *E. coli*. Despite the importance of differences in the details of structure, lipid A is the least variable of the three regions of LPS.

Core

The polysaccharide core region of LPS is subdivided into an inner and an outer region. Working out from the lipid A, the inner core includes the linking structure common to all LPSs. The unusual sugar providing this linkage is characteristic of LPS, 3-deoxy-D-*manno*-oculosonic acid, which is commonly abbreviated to KDO (reflecting the older name of 2-keto-3-deoxyoctonic acid). In most (but not all) gram-negative bacteria KDO is linked to another unusual sugar, heptose, which is in turn linked to the outer core. A second and sometimes a third KDO may be linked to the first KDO as a side branch. The sugars of the outer core are typically common hexoses, including glucose, galactose, glucosamine, and galactosamine.

The core influences the properties of the lipid A. Lipid A separated from the core is generally less toxic than that attached to the core. Some species of bacteria contain only a core polysaccharide and no repeating oligosaccharide region. These include the highly pathogenic *Bordetella pertussis*, *Neisseria meningitidis*, and *N. gonorrhoeae*. Rough mutants (so called because of the rough appearance of their colonies) of other species, notably *Salmonella*, are unable to synthesize the repeating oligosaccharide (Ra mutants). Other mutants are unable to synthesize not only the oligosaccharide, but also increasing portions of the core (Rb to Re mutants). These mutants have proved to be invaluable tools in the elucidation of structure of the core and the roles of its components.

Repeating Oligosaccharide

The repeating oligosaccharide is also referred to as the O-specific chain (O for oligosaccharide) and is the principal antigenic region of LPS. The repeating oligosaccharide region is highly variable, even between strains of the same species. The various serotypes of a species are typically determined by the different antigenic properties of their repeating oligosaccharides. The region consists of repeating groups of a small number of sugars; some strains only have one sugar represented, others have up to eight. The number of repeats of the oligosaccharide may be as high as fifty. There are many different sugars that may make up the oligosaccharide and multiple ways in which they may be linked. Consequently, there are an enormous number of potential conformations and a great deal of variability is observed in nature.

The repeating oligosaccharide, together with the core region, is hydrophilic and serves as the solute carrier of LPS. It is also reported that the repeating oligosaccharide downregulates the endotoxicity of LPS (5). Free LPS is not part of a living cell membrane. It was derived from a dead cell, or released during cell growth and division, or because the LPS has been purified, as in the case of a standard endotoxin preparation. Some free, purified LPS preparations are rather insoluble and must be solubilized to exhibit full toxicity (6).

Properties of LPS

The size or molecular weight of individual subunit of a particular LPS depends on its structure. LPSs with long repeating oligosaccharide chains are significantly larger than those with shorter chains or no oligosaccharides. Having said this, the molecular weight of a typical LPS subunit is about 10,000 Da, but may be less (down to a few thousand daltons) or more, depending on the length of the repeating oligosaccharide chain. However, endotoxin rarely occurs as individual subunit. From the discussion of structure, it is clear that LPS is amphiphilic. That is, one end of the structure is hydrophobic (the lipid A) and the other (the polysaccharide) is hydrophilic. Consequently, in aqueous solution the tendency is for the lipid A to aggregate while the repeating oligosaccharide is exposed to the aqueous medium. The conformation of aggregations of LPS depends on the details of the structure of the lipid and on the chemical nature of the medium. Structures include micellar, hexagonal, lamellar bilayers and ribbons, and cubic arrangements. Native (naturally occurring) endotoxin, which is the potential contaminant of healthcare products, is not a highly purified LPS preparation. The conformation of native endotoxin, with its associated proteins and other non-LPS membrane fragments, differs again and tends to be less ordered, but it will typically be present as aggregates.

Environmental factors that influence the aggregation state of endotoxin include temperature, pH, salts (particularly divalent cations), surfactants, bile salts, and proteins. Changes in the bioactivity of endotoxin have been reported with accompanying changes in aggregation state (as measured by sedimentation coefficient) (6). The notion that aggregation of endotoxin is required for its activity, including endotoxicity, has been advanced (7,8), while others have maintained that it is the individual subunits that are the active form. Suffice it to say that neither the aggregation state of endotoxin nor its activity is fixed. Both can change with chemical conditions and the input or the removal of energy. It is as well for those performing BETs to bear this in mind. Further, different endotoxins may not behave in the same way when conditions change. Those performing endotoxin tests may encounter differences between the behavior of native endotoxin in a sample and that of the purified endotoxin (LPS) of the standard preparation. When this occurs, it will be necessary to determine test conditions under which both endotoxins behave in a similar manner.

In addition to being amphiphilic, endotoxin carries a net negative charge, at least in aqueous solutions of pH 4 or higher. The negative charge prevents the degree of aggregations that might otherwise occur as like charges repel each other, countering the tendency of the hydrophobic lipid moiety of the structure to aggregate. This explains, at least in part, the mechanism by which cations in the medium enable formations of greater aggregations. Cations neutralize the negative charges and LPS tendency to repel one another. Divalent cations can neutralize the negative charges of two adjacent subunits and form cationic bridges linking the two, further promoting aggregation.

Finally, endotoxin is very stable and is not readily destroyed. Normal sterilizing conditions are generally not sufficient to destroy endotoxin. This applies not only to dry heat and steam sterilization, but also to ethylene oxide (EtO) and γ-radiation. In the late 19th and particularly the early 20th century, after the importance of sterility was appreciated and the potential of injectable therapies was being investigated, the phenomenon of injection fever was commonly reported. The nature of the fever-causing agent (or pyrogen) was not known, but it was recognized that it withstood sterilization (9). This pyrogen was endotoxin.

Effects of LPS

The toxic effects of LPS (or endotoxin) include fever (a pyrogenic response), local Schwartzman reactivity (following subcutaneous injection of a small, relatively nontoxic dose of endotoxin, a second dose injected intravenously results in bleeding at the site of the original injection), disseminated intravascular coagulation, hypotension, tachycardia, shock, and lethal toxicity. It is the concern about these effects that has led to endotoxin limits for injectable products and medical devices that contact the blood or cerebrospinal fluid (CSF).

The effects of LPS on mammals are frequently mediated by cytokines. Upon exposure to LPS, macrophages produce a range of cytokines, notably interleukin (IL-) 1, IL-2, IL-6, and tumor necrosis factor. The cytokines in turn elicit a range of responses, the severity of which depends on the dose of LPS and the nature of the response. As seen in the Schwartzman

reaction, multiple exposures can lead to sensitization to LPS, but with appropriate doses and timing can also result in increased tolerance. In the case of the pyrogenic response, cytokines reach the thermoregulatory center in the hypothalamus region of the brain and stimulate production prostaglandin E_2 (PGE_2), which activates the hypothalamus and resets the body's thermostat resulting in elevated temperature, or fever. More recently, it has been reported that endotoxin binding with Toll-like receptor (TLR) 4 in the hypothalamus can result in PGE_2 production, eliciting a fever response without (or in parallel with) cytokines (10).

The pyrogenic response is usually the initial effect of exposure of humans to lower, but physiologically significant, doses of endotoxin. Thus, endotoxin is a pyrogen, a substance capable of inducing fever. The need for a test for pyrogens was initially met by injecting rabbits with the substance in question and monitoring the rabbit for a temperature rise, that is, for fever. The test was developed in the early 1900s by Hort and Penfold (9) and refined and publicized by Florence Seibert in the 1920s (11). It was not until 1942, spurred by the entry of the United States into World War II and anticipation of the need for intravenous solutions in the battlefield, that the Pyrogen test was included in United States Pharmacopeia XII. In the pyrogen test, rabbits are injected with the test solution and monitored for temperature rise. Until the introduction of the LAL test in the 1970s, the pyrogen test was used to safeguard injectable therapies and critical medical devices.

The pyrogen test detects pyrogens from any source, not just bacterial endotoxins. This is in contrast with the LAL test, which is (generally) specific to endotoxin. Acceptance of the LAL test in place of the pyrogen test required a high degree of confidence that endotoxin was (and is) the pyrogen of greatest concern and the most likely pyrogen to occur in injectable therapies and medical devices. This illustrates the fact that the terms pyrogen and endotoxin are not synonymous.

Endotoxin exerts a direct influence in vitro on a wide range of cells other than macrophages. The effects include mitogenicity, inhibition of mitosis, morphological changes, and cytotoxicity. It is important that tissue culture media are not contaminated with an endotoxin concentration that is significant to the cells to be grown. The critical endotoxin concentration is very variable because the susceptibility of different cell types and cell lines to endotoxin varies substantially. In vitro fertilization is a special case of cell culture in which it is important to assure the absence of significant concentrations of endotoxin. In the presence of endotoxin, the success rates of fertilization, embryo implantation, and pregnancy are significantly reduced (12,13).

Because of the wide range of effects of endotoxin, it is important that the properties of a substance, such as a protein, be determined in the absence of endotoxin. Otherwise there is the danger that properties attributed to the substance are actually those of endotoxin.

Endotoxin Heterogeneity

The heterogeneity of endotoxin is apparent from the discussion above. The properties of endotoxin that have been discussed are generalizations and differ in degree between species or strains. They can also differ between cultures of the same organism grown in different media. Also, the properties of LPS preparations can be influenced by the extraction method and the degree of purification. Users of control standard endotoxin (CSE) for LAL testing may be aware that different batches of the same endotoxin preparation can have different potencies when measured with a given lot of LAL reagent. Potencies of CSEs are discussed in more detail later in this chapter.

Differences in chain length of the repeating oligosaccharide affect the solubility of the endotoxin in water. Solubility has been shown to influence the pyrogenicity of the endotoxin (6). Also, different endotoxins vary in potency in the pyrogen test and especially in LAL test (14). The differences in pyrogenicity in humans between endotoxins from different species are well illustrated by the classic studies from Greisman and Hornick (15), data from which are presented in Table 1. Similar differences in potency between species are evident in the LAL test.

Pearson et al. (16) report that native endotoxins are less pyrogenic than purified standard endotoxins of equal LAL reactivity. These authors noted that this phenomenon serves as a safety factor for endotoxin tests conducted using the LAL reagent. Similarly, LPS extracted from *Pseudomonas aeruginosa* has been found to be half as potent as LPS from *E. coli* in a

Table 1 Human Threshold Pyrogenic Doses of Three Endotoxin Preparations

Source of endotoxin (species)	Threshold pyrogenic dose (ng/kg)
Salmonella typhosa	0.1 0.14
Escherichia coli	1.0
Pseudomonas sp.	50 70

Note: The threshold pyrogenic dose is the minimum dose of endotoxin required to elicit a fever. The smaller the dose required the greater the potency of the endotoxin preparation.

LAL test. In contrast, in a whole blood pyrogen test, *E. coli* LPS was approximately 1000 more potent than that of *P. aeruginosa*, which occurs commonly in water and is much more likely to contaminate parenteral products than *E. coli* LPS (17). Thus, it appears that LAL reagent might overreport the threat posed by some endotoxins.

Endotoxin Standards and Units

In the early days of endotoxin testing using LAL, results were initially reported in units of weight of endotoxin. However, results in units of weight are often not comparable because different endotoxins have different potencies. The activity 1 ng of one endotoxin preparation is not necessarily equivalent to that of 1 ng of a different preparation. Also, results reported in units of weight imply that the test is measuring the absolute amount (mass) of endotoxin present. This is misleading. A result reported in ng/mL means that the endotoxin present has a reactivity equivalent to the stated number of ng of the standard endotoxin preparation being used in the assay. It is not a measure of the mass of endotoxin detected.

Primary Standards

The variability of activity between different LPS preparations resulted in recognition of the need for endotoxin standards. To meet this need, the USFDA commissioned the preparation of a quantity of LPS, which was designated EC, from *E. coli* O113:H10 K negative (18). From this stock material a series of standard endotoxin preparations were produced and FDA standard EC-2 was the first to be disseminated. Importantly, the endotoxin unit was introduced with EC-2 and defined as 5 EU/ng, or 5000 EU per 1 μg vial. The endotoxin unit is a measure of activity (or potency) of endotoxin as measured in a LAL test, *not* a measure of mass. Two subsequent batches, EC-3 and EC-4, did not become established standards, but EC-5 did and was made publicly available as United States Pharmacopoeia (USP) Endotoxin reference standard (RS) lot F. The potency of EC-5 was double that of EC-2, at 10,000 EU/vial and the mass of LPS was not stated for this standard.

More recently, the USP was responsible for the production of USP Endotoxin RS lot G, which was filled by the National Institute for Biological Standards and Control in the United Kingdom. This standard was prepared from the same bulk EC preparation as the previous FDA standards and, like EC-5, contains polyethylene glycol and lactose as excipients. The USP has made some of this lot available to the FDA, who designated the standard EC-6. In addition, some of this standard was given to the World Health Organization (WHO), who established it as the second International Standard (IS) and assigned the potency in International Units (IU) of endotoxin, with 1 IU being equivalent 1 EU. Another portion of this material was given to the European Pharmacopoeia (EP) who designated it Biological Reference Preparation 3 (BRP-3), also with potency expressed in IU.

As consequence of work by a number of national and international bodies, there is now a single reference standard endotoxin (RSE) for the US, WHO, and EP. Interestingly, and sometimes confusingly, vials of this single standard bear four different labels and two different units. These are summarized in Table 2. These standards may be referred to as primary standards as they do not derive their activity from any other current standard (though USP lot G was compared with EC-5 in an unpublished collaborative study).

Table 2 The Four Presentations of the Current Primary Reference Standard Endotoxin

Organization	Designation	Units	Comments
USP	Lot G	EU	Available from the USP
US FDA	EC 6	EU	EC 6 is only available to licensed manufacturers for standardization LAL reagents.
WHO	IS 2	IU	Accepted by WHO in October 1996
EP	BRP 3	IU	Available from the EP

Given the harmonization of the US, WHO, and EP endotoxin standards, the question arises how to take advantage of this so that test results can be expressed in either EU or IU. Firstly, the USP, EP, and Japanese Pharmacopoeia (JP) endotoxins test chapters state that 1 EU = 1 IU of endotoxin. Thus results in EU and IU can be considered equivalent. Secondly, the LAL and endotoxin manufacturer's certificates of analysis commonly give the potency of the CSE in both EU/ng and IU/ng.

The primary endotoxin standard in Japan is a different preparation from that used for the standards in Table 2. The (Japanese) endotoxin unit has been set to be equivalent to 1 (US) EU. A case can be made for calling this a secondary standard as the potency was set with reference to the USP standard. However, as far as the JP is concerned, this is the primary standard. Unfortunately, as was the case with WHO first International Standard and the European BRP-2 before harmonization of these standards with the US standards, sometimes it has not been possible for users of this standard to confirm the labeled sensitivity of a given lot of gel-clot LAL reagent. The reason for this is that the assigned equivalent potency for the Japanese standard may be significantly different from the actual potency as measured with the particular lot of LAL reagent, even though the *average* potency for multiple determinations made with multiple lots of reagent indicates a one-to-one equivalency. Thus the sensitivity of a gel-clot reagent (which is labeled on the vial of reagent produced by FDA-licensed LAL manufacturers and is determined with US endotoxin reference standard) differs from the sensitivity measured with the Japanese standard.

If the labeled sensitivity of a LAL lot cannot be confirmed with the Japanese RSE (or with a CSE of known potency), the potency of the standard can be determined with reference to the USP RSE. Because the RSE is used to do this, sensitivity of the LAL reagent can be verified against the primary standard, and the procedure will indicate whether there is truly a potency discrepancy or some other problem with the test or reagents. Potency determination is discussed in detail in the section on essentials of LAL testing later in this chapter.

Secondary Standards (CSE)

The term secondary standard is used here to denote one that has been standardized with reference to a primary standard. The term control standard endotoxin is commonly used for such secondary endotoxin standards. A CSE may be defined as "an endotoxin preparation other than the RSE that has been standardized against the RSE." This definition is taken from the USP BET chapter prior to harmonization of the chapter with the JP and EP [which can be found in Appendix B to the 1987 FDA Guideline (2)]. This definition is still applicable. The most widely used CSEs are those supplied by the LAL reagent manufacturers. These are usually preparations of LPS from *E. coli*, such as *E. coli* O113:H10 or *E. coli* O55:B5, with or without additives or fillers.

To use a CSE, its potency (or activity) must be determined with reference to a primary standard. Such determinations are specific to the lot of LAL reagent used for the determination. The importance of determining a specific potency for each combination of CSE lot and LAL lot was recognized in the pre-harmonization BET, which stated "Calibration of a CSE in terms of the RSE must be with the specific lot of LAL reagent and the test procedure with which it is being used." The procedures for determining potency are discussed in detail in the section "CSE Potency Determination" later in this chapter.

LAL REAGENT: SOURCE AND DISCOVERY

LAL is an extract from the blood cells (amebocytes) of the horseshoe crab, *Limulus polyphemus*. LAL contains the proteins of the blood clotting mechanism and clots in the presence of endotoxins (and also (1→3)-β-D-glucans). Unlike the blood of mammals, which contains all of the components required for clotting, horseshoe crab blood requires the external stimulus from endotoxin (or glucans) to clot.

The proteins of the clotting mechanism are located in granules in the amebocytes and can be extracted in a lysate obtained from disrupted cells. The raw lysate obtained from the cells is then formulated into a LAL reagent for one of the endotoxin test methods. A LAL test is any method that uses LAL to detect endotoxins. The gel-clot method mimics in vitro the response of the blood of a horseshoe crab in vivo when it is exposed to endotoxin (either by injury or by gram-negative bacterial infection).

Discovery and History of LAL

The beginnings of the LAL test for bacterial endotoxins lie in the work of Frederick Bang in the 1950s with descriptions of blood clotting and a bacterial disease of the horseshoe crab and the observation that clotting was caused by gram-negative bacteria (19,20). In the 1960s Levin and Bang demonstrated that clotting is initiated by endotoxin and that the clotting components of blood are located in amebocytes (21 23). In demonstrating that the source of the clotting mechanism is the white blood cells (amebocytes), Levin and Bang described the method of producing LAL. They also described gel-clot and kinetic turbidimetric test methodologies. Their work led to interest in the potential of LAL reagent as a diagnostic tool. However, the clinical value of testing for endotoxin has not been clearly demonstrated and the LAL test is not widely accepted as a diagnostic for gram-negative sepsis (24). However, more recently, a glucan-specific LAL reagent based test of human serum has been introduced as an aid in the diagnosis of invasive fungal infections (25).

While the clinical utility of LAL reagents was first being studied, the potential of endotoxin testing with LAL as an alternative to the pyrogen test was also being investigated. The first proposal for use of LAL test as an alternative to the pyrogen test for injectable products (radiopharmaceuticals) was published in 1970 by Cooper and coworkers (26). (One of whom was Levin, who was simultaneously investigating clinical applications of the reagent he had discovered.) Other studies followed and the range of products to which the test was applied expanded to include biologicals, parenteral solutions, and drugs (27 30).

In contrast with the mixed results obtained in clinical studies, endotoxin testing of healthcare products with LAL reagents showed considerable promise. However, if it was to replace the pyrogen test, a major obstacle had to be surmounted. By definition, the pyrogen test detects any pyrogen. In contrast, the LAL test is only sensitive to one significant pyrogen, endotoxin. This raises the obvious question, how significant are pyrogens other than endotoxin? The answer is hardly at all, which has allowed the LAL test to replace the pyrogen test in the great majority of applications. However, the question has not gone away. Pyrogen tests are still used in some cases. The EP Guidelines for using the BET (31) recommend parallel testing by the (pyrogen) test and the BET for manufacturers seeking to replace a pyrogen test with the BET for a product for which the pharmacopeial monograph specifies the pyrogen test.

Various in vitro pyrogen tests have been described in which release of cytokines by various cell types (both natural and cultured) is measured by ELISA. Five of these methods were evaluated in a study conducted under the auspices of the US National Institutes for Health (32). It was concluded that while none of the methods can be considered a complete replacement for the rabbit pyrogen test for the detection of gram-negative endotoxin, they can be considered for detection of endotoxin in human parenteral drugs on a case-by-case basis, subject to validation for each specific product to demonstrate equivalence to the pyrogen test.

Important work establishing the LAL test as a valid alternative to the pyrogen test was conducted by a group of workers from Travenol Laboratories (the forerunner of Baxter) in the late 1970s and early 1980s. Mascoli and Weary (33) described the substantial amount of comparative data collected on LAL and pyrogen tests. As early as 1977 this group conducted over 28,000 rabbit tests and more than 143,000 LAL tests. In these tests, 37 samples failed to meet the endotoxin specification and four of these samples also failed the rabbit pyrogen test.

None of the samples that passed the LAL test failed the pyrogen test, that is, no false-negative LAL test results were obtained. It is worth noting that there were no official (USP) endotoxin limits at this time and there was no standard endotoxin. The Travenol researchers had to set their own limits and identify an appropriate endotoxin standard.

In addition to reporting on the test data of parenteral products, the Travenol group examined the results of seven marginally failing pyrogen tests (cumulative temperature rises ranged from 3.7 to 4.55°C). In a positive eight-rabbit pyrogen test not all of the rabbits necessarily show a fever. The authors considered all of the possible combinations of the 56 rabbits from the seven tests. They showed that, given the worst case grouping of rabbits, approximately 30% of the tests could have passed the pyrogen test. This served to illustrate the variability of response of the test animal, one of the disadvantages of the pyrogen test.

The Travenol authors also described an incident of human serum albumin (HSA) contamination. Batches of HSA, which had been tested and released by the pyrogen test, were found to be pyrogenic in humans. This led to a program to develop a LAL test for HSA. To do this, it was necessary to resolve an interference problem. (A LAL test was also desirable because HSA is antigenic and rabbits must be destroyed after a short test life.) Once a test had been developed, Travenol screened HSA lot retentions and two more potentially pyrogenic lots were identified and withdrawn from the market. As a result of this experience in which the pyrogen test had failed to fully protect public health, FDA required LAL tests on future lots of HSA and prohibited release of batches that tested positive in the LAL test, even if negative in the official (i.e., pharmacopeial) rabbit pyrogen test.

Mascoli and Weary concluded the following: endotoxins are the primary pyrogen of concern in parenteral products; a negative LAL test had never been observed when the pyrogen test was positive; the LAL test is more sensitive than the pyrogen test (the USP pyrogen test had allowed the release of some materials proved to be pyrogenic in humans and were subsequently shown to contain endotoxin in LAL tests). The considerable variability of the pyrogen test was noted in this work and in other publications from the Travenol group (34,35).

In 1988 the USP held an open conference on alternatives to in vivo tests. At this time many manufacturers of parenteral products and medical devices were routinely using LAL tests to either supplement pyrogen tests (primarily by in-process testing) or replace the pyrogen test as a release test. This was despite the fact that the great majority of monographs in the USP specified the pyrogen test for most injectable products. The FDA Guideline on the LAL test (2) had been published in 1987, and by the time of the 1988 conference the LAL test had proven itself be superior to the pyrogen test in most applications. The LAL test for endotoxins was a prime example of an in vitro replacement for an in vivo test. In the succeeding few years after the conference requirements for the pyrogen test in USP monographs for injectable products were changed to a LAL test as described in the USP BET chapter (36). The great majority of monographs for injectable products now specify the BET, not the pyrogen test.

The LAL Clotting Reaction

Levin and Bang first proposed a mechanism for the LAL test (21) and postulated that endotoxin activated an extract from the blood of *Limulus* (which they termed "pre-gel") and formed a gel-clot. A simple model for the clotting reaction was subsequently proposed (Fig. 3).

This model proved to be essentially correct but the clotting mechanism is actually more complex in two regards. First, it has been clearly demonstrated that the activation of clotting enzyme by endotoxin is not direct. Endotoxin activates factor C, the first in a series of serine protease zymogens, which in turn activates factor B. Active factor B then acts on the proclotting enzyme, which cleaves the substrate in a classic cascade type of reaction. The intermediate enzymes are important as they amplify the initial signal (the recognition of

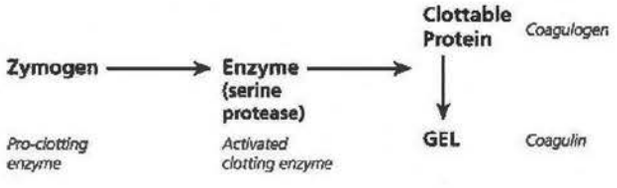

Figure 3 Early model for activation of the LAL clotting mechanism by endotoxin. *Source*: From Ref. 37. (Current names of components added in italics.)

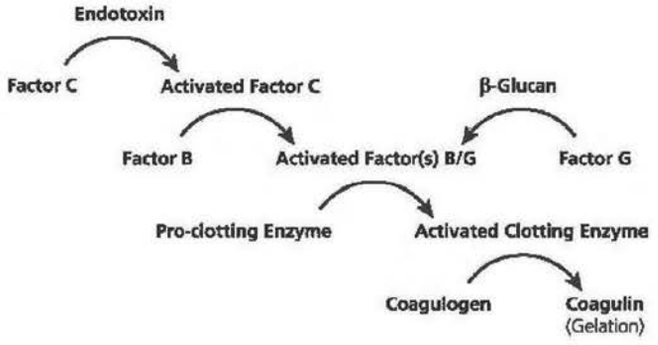

Figure 4 Clotting mechanism of *Limulus* amebocyte lysate. *Source*: From Ref. 38.

endotoxin by factor C) in a process analogous to a chain letter (if unbroken). The cascade reaction is the key to the extraordinary sensitivity of the LAL test. The activated clotting enzyme cleaves a peptide (peptide C) from the middle of the substrate, coagulogen (the accepted name for Levin and Bang's pre-gel). The two remaining peptides (A and B) remain linked by two sulfide bridges and reconfigure as the clotting protein, coagulin. Particles of coagulin coalesce and, when a sufficient concentration is reached, they coagulate to form a gel.

Second, the endotoxin initiated pathway is not the only route by which the clotting enzyme is activated. In a second pathway, (1→3)-β-D-glucan activates factor G, which acts directly on the proclotting enzyme (38). Activated clotting enzyme then cleaves coagulogen as described in the preceding text for the endotoxin pathway. LAL reagent is approximately 10-fold less sensitive to glucan than to endotoxin on a mass basis. This is attributable, at least in part, to the additional step in the endotoxin pathway that amplifies that reaction. The complete pathway is shown in Figure 4.

LAL Test Methodologies

Gel Clot Method

The gel-clot LAL test essentially mimics the clotting of *Limulus* blood in vivo. The end point of the test is a gel-clot, formed in a small reaction tube as opposed to a wound site on a horseshoe crab. The test is typically performed by mixing equal volumes of sample and LAL reagent and incubating the mixture under standard conditions of time and temperature (37 ± 1°C for 60 minutes ± 2 minutes). The sensitivity of gel-clot reagent is determined by the manufacturer using RSE and is labeled on the packaging. It is the minimum endotoxin concentration required to cause clotting. The term λ (lambda) is used to generically denote test sensitivity. Sensitivity is verified using dilutions of standard endotoxin, or at least by running a single positive control at a concentration that should always give a positive test result. Negative controls are also included with each test.

A gel-clot test is scored as positive if a clot forms, which withstands a smooth 180° inversion of the reaction tube. Otherwise it is negative, even if there is evidence of gel formation in the tube. A negative result means that the sample contains no detectable endotoxin, which is reported as a concentration of less than the labeled sensitivity of the reagent. A positive result means that the sample (or sample dilution) tested contains an endotoxin concentration of equal to or greater than the sensitivity of the reagent. The concentration of endotoxin in a sample can be quantified in a gel-clot assay by testing a series of twofold dilutions of sample. The dilutions are prepared in water that is free of detectable endotoxin. The greatest dilution at which a clot is formed is termed the *end point*. The endotoxin concentration in the sample is calculated by multiplying the dilution factor of the end point by the labeled sensitivity of the LAL reagent.

By convention, results of gel-clot assays are reported as single values, not ranges of $\geq X$ EU/mL but $< \frac{1}{2}X$ EU/mL. This is because of the error of the test, which includes the fact that the labeled sensitivity of the LAL reagent itself is determined by testing a series of twofold dilutions. No attempt is made to achieve resolution between twofold dilutions; such attempts

at greater precision are meaningless. It is generally accepted that the error of the test plus or minus a factor of two. Consequently, a result of 0.5 EU/mL should not be regarded as significantly different from either 1 EU/mL or 0.25 EU/mL. Acceptance of this variability is incorporated in the pharmacopeial endotoxins test chapters. When verifying the performance of a gel-clot reagent, the labeled sensitivity must be verified within a factor of two using standard endotoxin. Similarly, for positive and positive product controls (PPCs) a concentration of double the label claim sensitivity is used because it is recognized that the clotting may not occur at the sensitivity labeled on the reagent (though it may also clot at half the concentration of that labeled as the sensitivity). However, the reagent should always clot at an endotoxin concentration of double the labeled sensitivity. If it does not, the test is invalid and the reason should be determined.

Finally, in the pharmacopeial endotoxins test chapters the gel-clot method is the referee test, or the default compendial test, in the event of a dispute. In this context a "dispute" is typically an action initiated by a regulatory authority. Thus, resolution of a dispute is based on results from the gel-clot method unless another method is specified in the monograph for the product in question. Generally product monographs do not specify a test method (or "technique," to use the terminology of the pharmacopeia).

Photometric Methods

The harmonized USP, EP, and JP endotoxin test chapters address the turbidimetric and chromogenic LAL test methods under the common heading of Photometric Methods. This is a logical move because the fundamental principles of these methods are the same. There is no need to address them separately, as they are in the FDA Guideline. However, in the present chapter the methods are described separately because of their different chemistries. They are addressed together as photometric methods in the discussion of regulatory requirements.

In the LAL reaction, as the concentration of insoluble coagulin (the clotting protein) particles increases the reaction mixture becomes turbid. Reduction of light transmission due to the turbidity can be detected at a wide range of wavelengths, but is greater at shorter wavelengths. Because the initial steps of the cascade do not result in the production of insoluble coalescent protein, there is a time lag before turbidity start to increase. Levin and Bang (23) described how the rate of increase in turbidity [as measured by optical density (OD)] increased with endotoxin concentration. This phenomenon is the basis of the turbidimetric LAL test methods. Because turbidity development begins well before a solid gel is formed, this is a much more sensitive test than the gel-clot method.

The kinetic method is by far the most widely used turbidimetric technique. In it, typically the time taken to reach a specified level or threshold of turbidity is determined. This time is commonly called the "onset time." The higher the endotoxin concentration is in the sample, the faster the reaction and the shorter the onset time. There is an inverse log/log relationship between endotoxin concentration and onset time. Consequently, the sign of the slope of a plot of the log onset time against the log endotoxin concentration is negative (and so is the sign of correlation coefficient). Alternatively, the rate of the increase in turbidity may be determined, either as the V_{max} (the maximum rate of increase of OD calculated as a moving "boxcar" average over a relatively short time throughout the test) or V_{mean} (the mean rate of increase of OD over the test period). The slopes of standard lines for both V_{max} and V_{mean} are positive because these two parameters increase in value as the endotoxin concentration increases.

To perform a kinetic turbidimetric test, it is necessary to record the turbidity of the reaction mixture of each sample at regular intervals (no longer than about 1 minute) throughout the test while maintaining the reaction mixture at a stable temperature, typically 37°C. Prior to the availability of incubating, computerized instrumentation, this was a laborious procedure and not practicable for a routine assay, though Levin and Bang (23) did so. Since the 1980s and the advent of incubating tube and plate readers, kinetic test methods have become a practical methodology. A major advantage of the method is the wide range of endotoxin concentration that can be detected.

An alternative approach is to incubate the sample plus LAL reagent for an appropriate period and then to read the OD once at that end point. In the end point method, there is a linear relationship between endotoxin concentration and OD. There are three disadvantages of

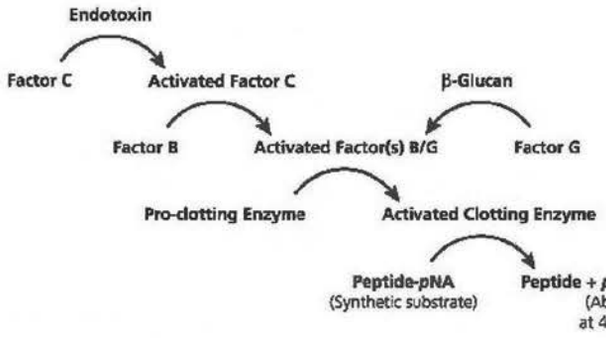

Figure 5 The chromogenic LAL reaction. *Source*: Modified from Ref. 39.

this method. First, for a given incubation time only a relatively narrow range of endotoxin concentrations can be detected; this range is generally about a factor of 10, commonly referred to as 1 log (base 10 assumed), such as 0.1 to 1 EU/mL. Higher endotoxin concentrations cannot be discriminated from one another because the reaction is largely complete and they are all approaching the same saturation point (i.e., the substrate is limiting). For lower endotoxin concentrations, the turbidity of the sample has not yet begun to increase significantly. Detection of higher or lower endotoxin concentrations requires shorter or longer incubations, respectively. Second, the development of turbidity cannot be stopped, so there is only one chance to read turbidity at the end point. The only advantage of this method is that an incubating reader is not required. The incubation can be performed using a heat block prior to reading the OD. However, this points to the third disadvantage, the operator must be available to read the test at the conclusion of the incubation (unless an incubating reader can be set to take the reading).

Kinetic turbidimetric assays are performed in either microtiter plates or in reaction tubes in a tube reader. A sensitivity of 0.001 EU/mL can be achieved in a tube reader, as opposed to 0.005 EU/mL in a microtiter plate reader. The greater sensitivity is due to the longer path length in a tube. Assuming a typical standard endotoxin potency of 10 EU/ng, 0.001 EU/mL is equivalent to 0.0001 ng/mL, or 0.1 pg/mL. This is 0.1 ppb, a high sensitivity for any assay.

In contrast with the turbidimetric method, which utilizes the endogenous chemistry of native LAL, in the chromogenic methods a synthetic substrate is added to the LAL reagent, either in the formulation of the reagent or by the user as the test is performed. The substrate is colorless and consists of a peptide to which a terminal chromogen, *para*-nitroanilide (*p*NA) is attached. The amino acid sequence of the peptide is recognized and cleaved by the clotting enzyme to release the *p*NA chromophore (now *para*-nitroaniline), producing a yellow-colored solution that, unlike the intact chromogen, absorbs light at a wavelength of 405 nm (Fig. 5).

In the chromogenic LAL test, the absorbance may be monitored in a kinetic mode and the onset time determined, just as it is in the kinetic turbidimetric method. Alternatively, an end point test may be performed. Unlike the turbidimetric reaction that cannot be conveniently stopped, the chromogenic reaction can be stopped by the addition of acetic acid and the absorbance of the chromogen measured. However, the end point method still suffers from two drawbacks: the limited detection range and the requirement of an operator to stop and read the test. Chromogenic assays are frequently performed in microtiter plates and achieve a sensitivity of 0.005 EU/mL, the same as turbidimetric assays in plates.

REGULATION OF ENDOTOXIN TESTING

The regulatory requirements for endotoxin testing are relatively straightforward, at least for those concerned with compliance in the United States, Europe, Japan, and countries that adhere to the regulations in these countries. The principal reference documents are the endotoxins test chapters in the USP, the EP, and the JP. Second to these documents is the US FDA Guideline on the LAL test, published in 1987. (This document includes an Appendix B that is the BET in USP XXI, which was the current USP at the time the Guideline was issued.) In 1991 FDA issued a follow-up guidance document that addressed kinetic turbidimetric testing of drugs and biologics. This document is incorporated in the version of the 1987 Guideline document available on the FDA website. In addition, in the EP under the section on General Texts, there is a chapter Guidelines (number 5.1.10) (31). The Guideline(s) and Guidance are

what the titles imply, guides, and they do not have the force of law. The pharmacopeial chapters do have the force of law, at least as far as release testing of products with monographs that specify an endotoxins test. Finally, there is a standard published jointly by the American National Standards Institute (ANSI) and the Association for the Advancement of Medical Instrumentation (AAMI) (40) titled "Bacterial Endotoxins Test Methodologies, Routine Monitoring and Alternatives to Batch Testing." This standard contains general information on endotoxin testing but is primarily aimed at testing of medical devices.

There are three core elements of regulatory compliance that are addressed in the pharmacopeial endotoxins test chapter and the FDA guidance documents. These elements are:

1. Verification of reagent performance/technician certification/laboratory qualification. Prior to testing product it must be demonstrated that the test is working and that it can be consistently performed by appropriately trained, certified technicians using endotoxin standards diluted in water.
2. Inhibition/enhancement Testing validation. Before testing a product to determine whether it can be released for use, it must be demonstrated that the test method will detect endotoxin in the product and, very importantly, that the test sample does not interfere with the ability to detect endotoxin.
3. Routine testing. Testing is conducted according to the method validated in step 2 and includes controls to once again assure that the test sample does not interfere with the ability to detect endotoxin. To meet the requirements of the test, the sample must contain less than the endotoxin limit.

These core elements should be borne in mind during the following review of the principal regulatory documents.

Pharmacopeial Endotoxins Test Chapters

As a result of a harmonization effort led by the JP, the USP and the EP published major revisions to the endotoxins test chapters in 2000. The JP revision was published in 2001. The EP and USP chapters became official on January 1, 2001, and the JP chapter became official on April 1 of the same year. (The English language version of the JP chapter became available in 2002 and is the only pharmacopeia available freely on the Internet (41).) The USP first published proposed changes to the BET in 2007. The final changes were published in USP 33 with an official (effective) date of October 1, 2010. Similar changes to the EP chapter were also published in 2007 and became effective on January 1, 2010 (42). The revisions did not alter the fundamental principles of the chapters.

The harmonized bacterial endotoxins chapters in the three pharmacopeia are very similar. There are some wording differences and some concepts are addressed in one document but not others. The introduction to the USP BET notes that portions of the chapter have been harmonized with the EP and/or JP and nonharmonized portions are marked. A summary of the content of the harmonized chapters follows.

***Important**: this summary is presented as a guide to the pharmacopeial chapters and as a basis for further discussion. It is not intended to be, and it should not be used as, an alternative to the pharmacopeial chapters. The chapter is always subject to revision and a summary will always omit some points and nuances that are present in the original document. The current version of the chapters should always be consulted when making compliance decisions.*

The chapter states that LAL is obtained from *Limulus* or *Tachypleus* and "manufactured in accordance with the regulations of the competent authority." The BET chapter cautions that "LAL reacts with some β-glucans in addition to endotoxins. Some preparations that are treated will not react with β-glucans and must be used for samples that contain glucans."

Three techniques (the gel-clot, turbidimetric technique, and chromogenic) are described in the initial, general section, not two (gel-clot and photometric). Testing may be conducted using any one of these techniques unless otherwise specified in the monograph. As noted in the discussion of the gel-clot method, in case of a dispute, the final decision is based on the gel-clot techniques unless otherwise specified in the monograph.

In the gel-clot methods, samples are tested in parallel with dilutions of an RSE. Endotoxin concentrations are determined using a sensitivity assigned to the reagent by the manufacturer, which is verified using the RSE. For the photometric methods, samples are tested and the endotoxin concentration is determined using a standard line constructed using a series of endotoxin RSE concentrations. The reagents do not have an assigned sensitivity. The chapter states the equivalence of the endotoxin unit and the international unit of endotoxin.

Regarding Apparatus and Glassware, the chapter states that glassware and materials should be depyrogenated and that commonly used minimum time and temperature settings are 30 minutes at 250°C for dry heat. Another footnote in the BET addresses validation and references USP chapter <1211> (43). An important caution states that plastics should be tested for contamination and interference.

The BET specifies use of USP Endotoxin reference standard (RS). (Since it was harmonized with the EP and JP chapters, the BET no longer mentions CSE. This is discussed further later in this chapter.) For details of reconstitution, use and storage of USP Endotoxin Reference Standard (Endotoxin RS) the user is referred to the "package leaflet" (i.e. instructions). Dilutions of RSE are prepared with Water for BET and should be used as soon as possible to avoid loss of activity by adsorption.

Under the heading "Preparatory Testing," two requirements are stated that must be met before official test can be performed. These are the first two essentials of LAL testing stated above, the first of which is the confirmation of LAL reagent sensitivity. It should be noted that this actually only applies to the gel-clot method as photometric reagents do not have assigned sensitivities. However, later in the chapter the requirements for photometric reagents are given. Secondly, the test performance must be validated in the presence of the sample under test by performing the test for interfering factors to demonstrate that the sample does not interfere with the detection of endotoxin.

Preparation of sample solutions is discussed. Drugs are dissolved or diluted while medical devices are extracted, both in Water for BET. The chapter states that other solutions may be used, but gives no further guidance as to what type of solutions these might be or what type of validation may be required for their use. The pH of the reaction mixture (sample + LAL) must be in the range specified by the LAL manufacturer, which the chapter notes is usually 6.0 to 8.0. This is an important point. If out of range, pH can be adjusted with acid, base, or suitable buffer as recommended by the lysate manufacturer. Buffers should be validated as free of detectable endotoxin and interfering factors.

An important parameter discussed is the maximum valid dilution (MVD). The MVD is the greatest dilution of the product at which the endotoxin limit can be detected. If a test is conducted at a dilution greater than the MVD, it cannot be stated that the sample passes the test; it clearly fails if any endotoxin is detected. The equation for calculation of the MVD is given:

$$\text{MVD} = \frac{\text{Endotoxin limit} \times \text{conc. of sample}}{\lambda}$$

where λ is the "sensitivity of the test".

The sensitivity of gel-clot reagents is labeled on the packaging. For photometric methods the sensitivity is the lowest concentration on the standard curve and is therefore flexible, within limits, and determined by the user when the range of the standard curve is selected. MVDs are discussed more fully in the section of this chapter on testing drug products.

The method by which endotoxin limits are established for drug products is described in the BET, and an equation is given for the calculation of the endotoxin limit for an individual drug product. Details regarding endotoxin limits are given in a footnote in the USP BET. The equation is discussed in detail in the section on testing drugs and biologics. Endotoxin limits for medical devices are not addressed in the pharmacopeial endotoxins test chapters, but are given in USP chapter <161>, "Transfusion and Infusion Assemblies and Similar Medical Devices" (44).

Requirements for the Gel Clot Technique

The section on the Gel-Clot Technique begins by stating that the endotoxin concentration required to cause clotting of the reagent under standard conditions is the labeled sensitivity of the LAL reagent, which is determined by the manufacturer. In the preparatory testing section

specific to gel-clot techniques, the first requirement is confirmation of labeled sensitivity (λ). This is performed using at least one vial of reagent for each new LAL lot or when test conditions change. Four standard endotoxin concentrations are tested in quadruplicate with negative controls. The endotoxin concentrations are 2λ, λ, $1/2\lambda$, and $1/4\lambda$. The test is performed by mixing equal volumes of standard endotoxin and LAL reagent (such as 0.10 mL aliquots). The reaction mixture is incubated according to the LAL reagent manufacturer's directions (usually at $37 \pm 1°C$ for 60 ± 2 min). Reading of the test is described, notably the requirement to smoothly inverting each test tube through 180° to determine whether a firm gel-clot has formed that withstands inversion, which defines a positive test. The lowest standard concentration ($1/4\lambda$) must test negative for the test to be valid. The end point is the last positive test in a series of decreasing concentrations of endotoxin standard (or sample). The geometric mean of the four end points must confirm labeled sensitivity, expressed in EU/mL, within a factor of two.

The second requirement for preparatory testing is the test for interfering factors. This requires confirmation of label claim in quadruplicate using endotoxin RS added to test sample at a dilution less than the MVD that contains no detectable endotoxin. In parallel with this, label claim is verified by testing in at least duplicate a series of standard endotoxin concentrations diluted in water. Though not stated explicitly in the chapter, the purpose of this is to demonstrate that the ability of the test to detect endotoxin in product (or a specified dilution thereof) is not significantly different from that in water. The pharmacopeial endotoxins test chapters use a table to explain test setup. If interference cannot be overcome at a product dilution less than the MVD, the MVD may be used to allow further dilution to overcome the interference.

In order for the test to be valid, the sample dilution to which no endotoxin has been added and the negative controls must test negative. The geometric means of the standard endotoxin concentrations in both water and in the product must confirm the labeled sensitivity of the LAL reagent.

The BET allows for interference to be overcome by treatment such as filtration, neutralization, dialysis, or heating. Such treatments must be validated by adding endotoxin to the sample, performing the treatment and then demonstrating that endotoxin can be recovered and is not removed by the treatment.

After the preparatory testing section, the pharmacopeial chapters describe the procedure for a gel-clot limits test, which is a positive/negative test at a particular endotoxin concentration. A positive limits test indicates that the sample being tested contains an endotoxin concentration of at least the sensitivity of the test. It gives no information about what that concentration might be. (Endotoxin concentrations are determined by performing an assay, which is described in the next section of the BET.) It is implicit in the wording of limits test that, when performed at the MVD, it is a pass/fail test at the endotoxin limit.

The procedure for the limits test is described in a table and may be summarized as follows. The specimen is tested at the dilution (not greater than the MVD) used in the test for interfering factors (and as treated in that test if applicable). The following controls are required: negative controls, positive controls consisting of an endotoxin concentration of 2λ made up in Water for BET and positive PPCs, which consist of product at the test concentration containing a standard endotoxin concentration of 2λ. All conditions (sample and controls) are tested in duplicate. A full series of standard endotoxin concentrations is not required.

In order for the test to be valid, both replicates of the negative controls must test negative (not clot) and both replicates of 2λ positive control and of the 2λ PPC must clot. To meet the requirements of the test, the test must be valid and the two replicates of the sample (or sample dilution) must test negative. The BET states that the test should be repeated if one replicate of the sample tests positive and the other negative. The sample does not meet the requirements of the test if any of the replicates test positive in the retest. If a positive result is obtained for the product at a dilution less than the MVD, the test may be performed at a dilution not to exceed the MVD.

The quantitative gel-clot test is used to assay the endotoxin concentration in a sample. Like the limits test, the test setup is described in a table. A series of sample dilutions of sample "not to exceed the MVD" are tested. A PPC, the same as described for the limits test, is included for the first dilution tested only. The BET requires inclusion of a full series of standard endotoxin concentrations and a negative control. Like the limits test, the assay is conducted in duplicate.

For a test to be valid, the negative controls must be negative, the end point of the series of endotoxin standards must confirm labeled sensitivity within a factor of two and the PPC must test positive. To calculate the endotoxin concentration for each replicate series of dilutions of the sample, the end point dilution factor is multiplied by the lysate sensitivity. The geometric mean of the endpoint endotoxin concentrations for the two replicate series is then calculated. The sample meets the requirements if the geometric mean endotoxin concentration of the sample is less than the limit in the monograph. If none of the dilutions of the sample test positive, the result is given as less than the concentration that would have been reported if the end point had been at the first (lowest or least) dilution of product.

There is a difference between the wording of BET and the wording for endotoxin limits in the great majority of product monographs in the USP. Most monographs state that the substance shall contain *not more than* a particular endotoxin limit. The BET states that the article (i.e., the product sample) meets the requirements of the test if it contains *less than* the endotoxin limit. This is consistent with the requirement for the limits test in which the sample must test negative at the MVD. The more conservative (stringent) specification (which is that in the endotoxins test chapter) should be adopted.

Requirements for the Photometric Quantitative Techniques

Photometric test methods are outlined in the introduction to the "Photometric Quantitative Techniques" section of the pharmacopeial chapters; both end point and kinetic approaches to the turbidimetric and chromogenic assays are addressed. Tests are carried out at the incubation temperature recommended by the LAL manufacturer, usually 37 ± 1°C.

Preparatory testing for the photometric techniques serves essentially the same purposes described for the gel-clot method: first, to verify that the test is performing properly and, second, to demonstrate that sample material being tested does not interfere with the detection of endotoxin. Unlike the gel-clot method, there is no labeled sensitivity to confirm for the first of these two requirements. The test is performed for each lot of LAL reagent and if there is any change in conditions that are likely to influence the test result.

To demonstrate that the test is performing properly, standard curve criteria are verified by testing at least three standard endotoxin concentrations in at least triplicate according to the recommendations of the LAL manufacturer. If the range of endotoxin concentrations exceeds two logs (i.e., a factor of 100 base 10 is assumed), additional standards are required to bracket each log increase in range. A standard line is constructed and the absolute value of the correlation coefficient (lrl) must be at least 0.980. The absolute value of the correlation coefficient is specified because of the negative slope and r value of the standard lines for most kinetic assays. It is notable that, unlike the gel-clot method, there is no check against manufacturer's criteria or other external standard.

The interfering factors test for the photometric techniques is described in another table. A series of at least three standard endotoxin concentrations are tested to construct the standard curve as was described in the verification standard curve above. The same considerations regarding the range of the curve applies, but a minimum of two replicates are required, not three. The lowest of the standard endotoxin concentrations in the series is designated λ. This is the lowest concentration that can be quantified and is therefore the sensitivity of the test; it is thus analogous to λ in the gel-clot method. Negative controls are also required. Samples are tested at a dilution not to exceed the MVD, "unspiked" and "spiked" with added endotoxin to give a PPC with a concentration at or near the middle concentration of the standard curve. (The terms "unspiked" and "spiked" are not used in the pharmacopeial endotoxins test chapters, but are commonly used in LAL testing.) Like the standard series, the negative control, sample, and PPC are all tested in at least duplicate.

Two requirements for test validity are described. First, the absolute value of the correlation coefficient of the standard curve generated must be greater than or equal to 0.980. Second, the endotoxin concentration of the negative control must not exceed the limit described in the instructions for the lysate reagent (or it is less than the endotoxin detection limit of the lysate reagent employed). The mean measured concentration of endotoxin in the PPC must be quantified within 50% to 200% of the known concentration after subtraction of any endotoxin in the unspiked sample. When the endotoxin recovery is out of the specified

range, the interfering factors must be removed as described for the gel-clot method. The treatment must be validated and shown to eliminate interference without loss of endotoxins. This is achieved by performing the assay described above (the test for interfering factors) on sample to which a known concentration of standard endotoxin has been added and which has then been submitted to the treatment.

One of the pleasures of the pharmacopeial endotoxins test chapters is the section on the procedure for testing by the photometric techniques, which consists of a single sentence that refers to the section on the interfering factors test. There is elegant simplicity to the procedure for the test being the same as that used for its validation.

The endotoxin concentrations are determined for each replicate of unspiked sample (i.e., with no added endotoxin, which is solution A in the table presented in the chapter) using the standard curve. In order for the test to be valid,

- The standard series must meet the requirements described under Verification Criteria for the Standard Curve, that is, the correlation coefficient, |r|, must be at least ≥ 0.980.
- The added endotoxin spike must be quantified within 50% to 200%
- Negative controls should not exceed the limit in the description of the LAL reagent used (i.e., as described in the instructions for use in the product insert).

The final section of the chapters, "Interpretation" states that to meet the requirements of the test the mean of replicates of the sample must contain less than the endotoxin limit when corrected for dilution and concentration. However, this statement should be interpreted in the context of current good manufacturing practice, which is discussed in the section on retesting later in this chapter.

It should be noted that if a standard operating procedure states that testing is performed according to the pharmacopeial method, close attention should be paid to the details of the procedure. For example, use of RSE (not CSE) is specified in the USP. Also, a full series of standards is required for every quantitative test, but not for the gel-clot limits test. Any deviations from the specifics of the BET should be stated.

The FDA Guidance Documents

While the harmonized endotoxins test chapters serve as the primary reference for LAL testing, the 1987 FDA "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" (2) ("the Guideline") is a valuable reference, despite its age. The Guideline contains additional information on points not addressed in the pharmacopeial chapters. The whole document is not summarized here, but key points are noted.

The Guideline states that a CBER (the FDA Center for Biologics Evaluation and Research) licensed reagent shall be used for validation, release, and in-process testing (the latter despite the title of the Guideline, which refers to end-product testing). Variability of the test laboratory should be assessed by having each analyst qualify the reagent and Appendix A is referenced for details. However, Appendix A is missing from the version of Guideline available on the FDA website. The USP BET preparatory testing sections on confirmation of sensitivity or Verification Criteria for the Standard Curve can be used for the gel-clot and photometric methods, respectively.

The prescribed limit for positive product control (PPC or spike) recovery for photometric methods is ±25%. This is now superseded by harmonized pharmacopeial endotoxins test chapters, which give a range of 50% to 200%. Similarly the endotoxin "spike" concentration in PPCs of 4λ is also superseded by endotoxins test chapters, which specify that the PPC contain a concentration equal to or near the middle of the standard endotoxin series.

The Guideline refers to the BET test for interfering factors as "inhibition and enhancement testing" and "validation." It states that at least three batches of each finished product should be tested for inhibition and enhancement and it gives guidance on when to repeat validation. When the LAL reagent manufacturer is changed, but not the test method, the inhibition and enhancement test should be repeated for one lot of product. What is not stated, but should be assumed, is that this recommendation is based on the assumption that the

degree of interference is likely to be similar with the two manufacturers reagents and that the test on a single lot will serve to verify this. However, it may not be true that interference is similar as reagents from different manufactures may have quite different formulations and are unlikely to have the same interference characteristics (45). Consequently, if the reagent manufacturer is changed and the requirements for the test for inhibition and enhancement are not met, appropriate steps should be undertaken to overcome the interference. As a new test method has been developed, it would be prudent to conduct inhibition and enhancement testing on three lots of product. The Guideline states that when the test method is changed, regardless of whether the manufacturer remains the same, validation should be performed on at least three lots.

Interestingly, the Guideline does not recommend revalidation for changes to the manufacturing process, formulation, source of an ingredient, or LAL reagent lot change. It is stated that the PPCs can be used to reverify validity. This is reasonable for changes in lot of LAL reagent (from a single manufacturer) and perhaps for changes in source of well-characterized chemical components. However, changes in formulation and manufacturing process have the potential for changing the chemistry of the sample and consequently its interference characteristics. The pharmacopeial endotoxins test chapters require validation for such changes, so they should be evaluated with the need for revalidation in mind, at least on a single lot of product. For the photometric methods, since the procedure for the test is the same as that for the test for interfering factors, this is quite straightforward and revalidation is a matter of appropriately documenting the procedure. The actual test is unchanged. For the gel-clot method, consideration should be given to performing the test for interfering factors since the limits test and the assay do not indicate enhancement.

For photometric methods, the Guideline includes provision for use of product standard curves that are addressed in the section "Test Method Development" later in this chapter.

For routine testing, the Guideline states that samples, standards, PPCs, and negative controls should be tested in at least duplicate, as do the pharmacopeial endotoxins test chapters. Sampling should be based on the manufacturing procedures and the batch size. A minimum of three units, taken from at least the beginning, middle, and end of the manufacturing run is recommended. Samples may be pooled for testing. (The same can be applied to samples for validation.)

For the gel-clot method, the Guideline allows that, once consistency has been demonstrated in the test laboratory, it is not necessary to run a standard series with every test. A full series should be included with the first test of the day and repeated if LAL reagent or endotoxin lot, or other test conditions change. This is different from the pharmacopeial chapters, which specify a positive control only for limits tests and a full standard series with assays. Negative controls are required for all tests.

The Guideline provides for the use of archived (or stored) standard curves for the kinetic turbidimetric method. An archived standard curve is a set of previously determined parameters (usually the slope and y intercept) that define the standard line used to calculate the endotoxin concentrations of unknowns. The curve is valid over the range of standard endotoxin concentrations used in its construction. Prior to using archived curves, the Guideline states that consistency of standard curves should first be demonstrated. For each test in which an archived curve is used, the Guideline specifies inclusion of a standard control and that the (presumably mean) measured concentration of the control should be within ±25% of the nominal concentration. This serves to confirm the validity of the archived standard curve. This same control should be run for cartridge test methods that rely on an archived standard curve.

Like the pharmacopeial endotoxins test chapters, the Guideline includes some provisions for retests and these are discussed below in the context of the FDA Guidance on Out-of-Specification (OOS) results of 2006 (46).

The FDA Interim Guidance of 1991 is specific to Human and Veterinary Drug Products and Biologicals (but apparently not to medical devices) and addresses Kinetic LAL techniques. This document is inserted in the middle of the online version of the 1987 Guideline. In the Guidance the requirement for spike recovery for PPCs is increased to ±50%, but, as stated for the Guideline, this is now superseded by the pharmacopeial endotoxins test chapters. In addition, a PPC concentration of 0.5 or 5 EU/mL is specified, depending on the endotoxin limit for in the dilution being tested. Again this is superseded by the pharmacopeial chapters.

The document also includes additional guidance on archived standard curves. It specifies that an archived curve should be constructed from the data points of a minimum of three standard series tested over three consecutive days.

Control Standard Endotoxins

Finally, before leaving this review of regulatory documents, it is notable that there is no longer any mention of CSE in the BET. CSEs are addressed in Appendix C to the FDA Guideline (as well as in Appendix B, which is the pre-harmonization BET from USP XXI). The removal of mention of CSE from the USP caused some concern among LAL users. However, the issue of CSEs was addressed in Pharmacopeial Forum 26(1), Jan Feb, 2000, when the proposed text of the harmonized chapter was published. The preamble to the proposed new BET chapter stated:

"The use of in-house standards shown to be equivalent to USP Reference Standards is permitted under the requirements for alternate methods in the General Notices. The CSE has thus been deleted because in-house standards have to be shown to be equivalent to the USP Endotoxin RS."

Thus, it is clear that the USP did not intend to change the status of CSEs or prevent their use. However, in a referee test, the USP reference standard should be used to assure full compliance with the BET. The term "in-house standards" used in Pharmacopeial Forum suggests standards that are made up in individual laboratories. CSEs provided by the LAL manufacturers are widely used throughout the industry. They are not made and used by a single laboratory or one organization. They are more than in-house standards, but they should be validated as alternatives to the USP Endotoxin Reference Standard. Consequently, alternate endotoxin standards shown to be equivalent to the RSE can be used for routine tests and their validation.

Documentation of the equivalence of the CSE to RSE is provided by LAL reagent manufacturer's certificates of analysis (Cs of A) that state the potency of the CSE relative to RSE. There is a long history of the acceptance of Cs of A by regulatory agencies of many countries. One option is to accept the certificate of analysis at face value. (It should be noted that for the gel-clot method, every successful confirmation of label claim using a CSE supports the potency stated on the C of A.) Another option is to perform testing on a limited number of lots of CSE to confirm that potencies given on certificates of analysis are correct and verifiable in the user's laboratory and thus justify accepting the potencies stated on Cs of A. Finally, the potency determination can be performed for every CSE/LAL lot combination, either in place of the potency on the C of A or to verify each C of A received.

TESTING DRUGS AND BIOLOGICALS

Endotoxin Limits, Maximum Valid Dilution, and Minimum Valid Concentration

Endotoxin limits are generally based on the threshold pyrogenic dose for endotoxin. This is the minimum amount of endotoxin that can be expected to elicit a pyrogenic response. The term K is used for generic endotoxin limits. The threshold pyrogenic dose and the value of K is 5 EU/kg of patient body weight per hour for parenterally administered drugs and therapies, other than those administered intrathecally [into the cerebrospinal fluid (CSF)]. Both a controlled clinical study (1) and field experience (47) demonstrate that a pyrogenic response, may be elicited by endotoxin at this very low dose. The endotoxin limit is more stringent for products that are administered intrathecally and the value of K is 0.2 EU/kg/hr. (Note: None of the pharmacopeial chapters state that K is expressed per hour. It is necessary to express the limit per hour if the maximum dose of the drug product is expressed per hour in order for the units to cancel out in the endotoxin limit equation.) From the generic values for K, product specific limits are calculated and these are given in product monographs in the USP. For products that have a dose expressed per person, the average human adult body weight is assumed to be 70 kg. (Note: An average human body mass of 60 kg is assumed in the JP (41).) Thus, the endotoxin limit on a whole body basis is 350 EU/hr for parenteral products that are not administered intrathecally.

The product-specific endotoxin limit for a parenteral product is based on the maximum dose of the product. The greater the dose of the product, the lower (or more stringent) the endotoxin limit per unit of product. In the pharmacopeial endotoxins testing chapters and in

the FDA Guideline, the formula given for calculating the endotoxin limit for a product is Limit = K/M

where,

K is the generic endotoxin limit in EU/kg/hr (5 EU/kg/hr for most products; 0.2 EU/kg/hr for intrathecally administered products)

M is the maximum bolus dose of product/kg. If the product is injected at frequent intervals or administered continuously, M is the maximum total dose administered in a single hour period. If the dose is expressed for whole (adult) body, divide it by 70 kg to give the dose per kg.

Note 1: The maximum dose, M, excludes the heroic dose that might be used under extraordinary circumstances.

Note 2: The 1987 Guideline states that the dose used should be the rabbit dose (as used in the USP pyrogen test) or the maximum human dose, whichever is greater. USP has changed the basis of calculation of endotoxin limits to human doses only.

The equation gives the endotoxin limit expressed per unit of product, where the units are those in which the dose is expressed (weight, volume, international unit, equivalents, etc.).

In the case of drugs administered per m^2 of body surface area, calculations of endotoxin limits are based on an average human adult surface area of 1.8 m^2. Given the whole body limit of 350 EU and an average human surface area of 1.8 m^2, the endotoxin limit expressed per square meter is 350 EU/1.8 m^2 = 194 EU/m^2. This value of 194 EU/m^2 is an absolute and is equivalent to a value of 5 EU/kg for K for products administered per kg, but it is not currently stated as such in any regulatory document. To calculate the endotoxin limit of a product with a dose expressed per square meter, divide 194 EU/m^2 by the maximum dose (M) to give an endotoxin limit per unit of product. This is a simplification of the approach described in the BET, which states that the dose per m^2 should be multiplied by 1.8 m^2 and divided by 70 kg to convert it into a dose per kg. The product-specific limit is then calculated as described above by dividing the 5 EU/kg/hr by the dose/kg/hr.

For radiopharmaceuticals, the BET and the FDA Guideline state that endotoxin limits are calculated using a variant of the K/M equation whereby the limit = $175/V$ (and for intrathecally administered radiopharmaceuticals the equation is $14/V$). In these equations, V is the maximum dose (on a whole body basis) in mL at expiration of the product. It should be noted that there is an error in these equations in that the units have been omitted. To give limits in EU/mL, the equation must be expressed as:

$$\text{Limit} = \frac{175\,\text{EU}}{V}$$

(and 14 EU/V for intrathecally administered radiopharmaceuticals).

The endotoxin limits for health care products do not account for a patient being given several drugs and/or solutions at once, and it had already been noted that the limit of 5 EU/kg/hr does not give a significant safety margin. Fortunately, most drug products and solutions are considerably cleaner than the allowable limit. Many companies have internal specifications that are tighter than the limit, particularly for in-process samples and materials, and these provide a safety margin. If a more stringent limit is set for a product and stated as a specification, then that is the specification against which the product must be judged. A product that exceeds that limit fails to meet specification, even if it contains less endotoxin than the pharmacopeial limit.

For some products, there are compendial limits that are not based directly on the K/M equation. The endotoxin limit for large volume parenterals (dose of at least 100 mL) is generally not more than 0.5 EU/mL. This limit has its origins in a rabbit dose of 10 mL/kg. The limit for water for injection (WFI) and sterile WFI (SWFI) is less than 0.25 EU/mL (USP/EP).

Product-Specific Endotoxin Limits

When faced with an unfamiliar product, the product monograph in the appropriate pharmacopeia should be consulted. The maximum dose for the product should be determined from the product insert and the limit should be calculated for that dose. It is possible that the limit in the monograph was calculated from a different or incorrect dose, or that a mistake has

been made in the calculation. As a general rule, in such cases the more conservative (more stringent or lower) endotoxin limit should be adopted.

Endotoxin limits in pharmacopeial monographs are stated per unit of active pharmaceutical ingredient (API). It is important to understand that this is actually the limit for the whole finished product. Similarly, endotoxin limits calculated from the dose of API is the limit applied to the whole finished product.

Calculating Endotoxin Limits

Consider a parenteral product (with a nonintrathecal route of administration) with a maximum human (whole body) dose of 1.0 g. Using the equation given in the pharmacopeia and in the FDA Guideline,

$$\text{Limit} = \frac{K}{M}$$

First the dose must be converted into a dose per kilogram of body weight:

The dose per kg = 1 g/70 kg = 0.0143 g/kg = 14.3 mg/kg.
The endotoxin limit = K/M = 5 EU/kg/14.3 mg/kg = 0.35 EU/mg

The sensitivity of the LAL test used to test the product is expressed in EU/mL. To give an endotoxin limit in the same units as the test (unless the limit is already expressed in EU/mL), the endotoxin limit per unit of product is multiplied by the concentration of the product (sometimes referred to as the potency). To continue with the above example, if the concentration of product is 100 mg/mL, or if a solution of product is prepared to give 100 mg/mL, the limit is 0.35 EU/mg × 100 mg/mL or 35 EU/mL. This limit is specific to a product concentration of 100 mg/mL.

For a product with a dose of 1 g/m^2 and a concentration of 50 mg/mL, the endotoxin limit = 194 EU/m^2/1 g/m^2 = 194 EU/g (or 0.194 EU/mg). (Using the equation presented in the USP is a little less straightforward but gives the same result.) The limit expressed per mL = 0.194 EU/mg × 50 mg/mL = 9.7 EU/mL.

For a radiopharmaceutical product with a (whole body) dose at expiration of 7 mL, the endotoxin limit = 175 EU/7 mL = 25 EU/mL.

Calculating the MVD

Many drug products interfere with the LAL test. However, the fact that the LAL test is usually more sensitive than is necessary to detect the endotoxin limit (and sometimes much more sensitive) allows products to be diluted to overcome interference. In an early study conducted by FDA (30), of 333 products test dilution was effective in overcoming the interference for 236 products. Dilution is the most widely used means of overcoming interference. However, there is a limit to the amount by which a product can be diluted and the endotoxin limit can be detected. That limit is the MVD, a concept addressed in the pharmacopeial endotoxins test chapters and introduced in that section above. At the MVD, the endotoxin limit for the product dilution is equal to the sensitivity of the test. Valid testing cannot be conducted at dilutions greater than the MVD because in a sample contaminated at the limit concentration the endotoxin cannot be detected.

The concept of the MVD is quite intuitive. Consider a product with an endotoxin limit concentration of 25 EU/mL that is tested with a gel-clot reagent with a sensitivity of 0.25 EU/mL. Clearly the reagent is 100× more sensitive than is necessary to detect the limit concentration. Consequently, the product can be diluted by a factor of 100 and endotoxin at the limit concentration can still be detected. The MVD is 100.

The formula for calculation of the MVD as given in the section on the Pharmacopeial Endotoxins Test Chapters above is:

$$\text{MVD} = \frac{\text{Endotoxin limit product concentration}}{\lambda}$$

where λ is LAL test sensitivity.

If the endotoxin limit is already expressed in EU/mL, the equation may be simplified to

$$\text{MVD} = \frac{\text{Endotoxin limit (EU/mL)}}{\lambda}$$

The MVD is a unitless dilution factor.

As an example, for the hypothetical product with a limit expressed as 0.35 EU/mg tested using a LAL reagent with a sensitivity (λ) of 0.25 EU/mL:

$$\text{MVD} = \frac{0.35\,\text{EU/mg} \times 100\,\text{mg/mL}}{0.25\,\text{EU/mL}}$$

If the limit is expressed as 35 EU/mL (see above):

$$\text{MVD} = \frac{35\,\text{EU/mL}}{0.25\,\text{EU/mL}} = 140$$

Note that MVD increases as the sensitivity of the method/reagent increases. If the sensitivity of the LAL test is increased to 0.125 EU/mL for the above product:

$$\text{MVD} = \frac{35\,\text{EU/mL}}{0.125\,\text{EU/mL}} = 280$$

Thus, the MVD of a product can be increased by using a more sensitive gel-clot reagent or by using a lower range standard curve for photometric methods.

Calculating the MVC

A parameter directly related to the MVD is the minimum valid concentration or MVC and is discussed in Appendix D to the FDA Guideline, but not in the pharmacopeial endotoxins test chapters. The MVC is the product concentration at the MVD. It is the lowest concentration of product at which the endotoxin limit can be detected. The MVC is inversely related to the MVD because the greater the dilution of the product, the lower the concentration.

The formula for calculation of MVC is

$$\text{MVC} = \frac{\lambda}{\text{Endotoxin limit}}$$

For example, for the product with an endotoxin limit of 0.35 EU/mg, if the LAL sensitivity is 0.25 EU/mL:

$$\text{MVC} = \frac{0.25\,\text{EU/mL}}{0.35\,\text{EU/mg}} = 0.71\,\text{mg/mL}$$

Unlike the MVD calculation, the initial concentration of the product is not a parameter in the equation. Thus, the MVC is constant for a given LAL test sensitivity, while the MVD changes with the product concentration.

The conversion of MVC to MVD is described in Appendix D of the FDA Guideline and is accomplished by dividing the product concentration by the MVC. For example, continuing with the example from above,

$$\text{MVD} = \frac{\text{Product concentration}}{\text{MVC}} = \frac{100\,\text{mg/mL}}{0.71\,\text{mg/mL}} = 140$$

MEDICAL DEVICES

Introduction

To test a medical device, an aqueous extract of the medical device must be prepared because endotoxin tests can only be performed on aqueous samples. The USP BET states that extracts of medical devices should be prepared using Water for BET (formerly and commonly known as

LAL Reagent Water, or (LRW)) or other aqueous solutions. In the EP and JP bacterial endotoxins chapters, there is no mention of medical devices. Procedures for preparing extracts of medical devices are given in USP chapter <161>, "Transfusion and Infusion Assemblies and Similar Medical Devices." This chapter applies to a wide range of devices but orthopedic products, latex gloves, and wound dressings are explicitly excluded from the scope. Slightly different extraction procedures are given in the FDA Guideline on LAL testing and these are echoed in the standard ANSI/AAMI ST72:2002.

Endotoxin Limits for Medical Device Extracts

Generic endotoxin limits specified in USP chapter <161> are 20 EU/device for most devices labeled as nonpyrogenic and 2.15 EU/device for devices that contact the CSF. The formula given for the calculation of a limit specific to the extraction volumes in EU/mL is

$$\text{Endotoxin limit} = \frac{K \times N}{V}$$

where,

K is the endotoxin limit per device (e.g., 20 EU/device for devices that do not contact CSF)
N is the number of devices to be tested
V is the total volume of extract or rinse (i.e., extract volume per device multiplied by the number of extracts pooled).

Once an endotoxin limit has been determined, the concept of the MVD can be applied as described for drug products. The MVC is less useful for medical devices and is rarely used.

The formula for endotoxin limits does not account for unequal distribution of endotoxin contamination between the devices. For example, if 10 extracts of 40 mL are pooled to give 400 mL, the endotoxin limit is 0.5 EU/mL per the above equation. The total amount of endotoxin in the extract must reach 200 EU before a test failure is recorded at 0.5 EU/mL. It is quite possible that all of this endotoxin could have come from a single device. Consequently, there is a discrepancy between the stated limit 20 EU/device for a single device and a de facto limit of 200 EU/device when 10 extracts are pooled, a point that has not been widely acknowledged. In the introductory background section, the FDA Guideline recognizes the potential for unequal distribution of endotoxin on medical devices and that extraction procedures are unlikely to be 100% efficient. The endotoxin limit for medical devices is apparently more stringent than that for drugs (200 EU/person vs. 350 EU/person) for this reason.

Sampling Medical Devices for Testing

Sampling procedures for medical devices are based on lot size but there are slight differences between the FDA Guideline and USP <161>. The USP specifies testing 3% of the lot up to a maximum of 10 devices and no fewer than three devices. The FDA Guideline states that for lot sizes of less than 30 devices, 2 devices should be tested. For lot sizes of between 30 and 100 units, test three devices, and for lot sizes of greater than 100, test 3% of the lot up to a maximum of 10 devices.

Extraction Procedures

Extraction procedures in USP chapter <161> are simple and clear. Not less than three and not more than 10 devices are rinsed or soaked at controlled room temperature for 1 hour in LRW that has been heated to 37 ± 1°C. For devices labeled "nonpyrogenic fluid pathway," the pathway is flushed for 1 hour at controlled room temperature, again using the extraction solution that has been heated to 37 ± 1°C. It may be necessary to cut up or dissemble devices before extraction but this is not stated in chapter <161>. It is stated that "Extracts may be combined, where appropriate"; combining extracts is usual for testing medical devices.

Extraction procedures in the Guideline are the same as those in the AAMI/ANSI standard ST72 but are different from the USP. For devices being flushed, the rinse solution should be held in the fluid path for one hour at room temperature (>18°C). To perform extraction, the minimum extraction time should be 15 minutes at 37°C or one hour at room temperature (>18°C). Of these procedures, the one given in USP Chapter <161> includes both the maximum time and temperature. It also has the advantage of being a compendial procedure and therefore does not require validation.

The question of validation of the effectiveness of extraction procedures was addressed by the AAMI Microbiological Methods Committee, a group that included a representative from FDA. Validation of an extraction procedure requires adding a known amount of endotoxin to a device (usually drying it) and then demonstrating recovery using the procedure. The task group's report (48) concluded that validation of efficiency of endotoxin recovery should not be included in the ANSI/AAMI ST72 (40) standard.

The Guideline allows different devices of similar chemical (i.e., material) composition to be grouped for inhibition or enhancement testing. The devices selected for validation should be those with the largest surface area exposed to the body or the fluid for administration to a patient.

Manufacturers of pharmaceutical product can draw from the provisions for testing medical devices to meet the pharmacopeial endotoxins test chapter requirement that plastics be tested for contamination and interference. These must be extracted prior to testing in a manner similar to medical devices. However, appropriate limits should be set. The equation given for medical devices above is usually not applicable and could allow for unacceptable levels of endotoxin contamination. Finally, the Guideline states that "liquid devices should be more appropriately validated and tested according to the requirements for drugs by taking the maximum dose per kilogram of body weight into consideration."

ESSENTIALS OF ENDOTOXIN TESTING—A PRACTICAL APPROACH

In the introduction to the discussion of the pharmacopeial endotoxins test chapters, three essentials of endotoxin testing were identified. In addition to these, there are two other important elements. One of these (the second in the list below) is determination of CSE potency. This topic was introduced in the section on secondary standards (CSEs) and discussed in the section on the FDA Guideline.

The second additional element (number three on the list below) is preliminary testing of samples for test method development. This is a very important element, but it is not addressed in any of the regulatory documents. If method development is performed correctly, validation (the test for interfering factors) and subsequent routine testing should be trouble free.

Together, the five elements are:

1. Verification of reagent performance, technician certification, laboratory qualification
2. CSE potency determination, if necessary (not addressed in the pharmacopeia)
3. Preliminary testing (not addressed in any regulatory document)
4. Inhibition/enhancement testing - validation
5. Routine testing

Practical aspects of the application of these essentials are discussed in turn.

Verification of Reagent Performance, Technician Certification, and Laboratory Qualification

As was stated in the review of the regulatory requirements, it must be demonstrated that each lot of reagent is performing to specification prior to performing any tests of product. Also, the Guideline states that analysts must be qualified, which is a requirement of the good manufacturing practice (GMP) regulations, and that the variability of the testing laboratory should be assessed. The results for technician qualification can be compared to determine the variability of the test laboratory. These qualifying tests are all performed using the same procedure.

For the gel-clot method, this is achieved by confirming the labeled sensitivity of the LAL reagent by testing a series of four standard endotoxin concentrations with concentrations of 2λ, λ, $1/2\lambda$, and $1/4\lambda$ (and negative controls) in quadruplicate. For example,

Standards		2λ	λ	$1/2\lambda$	$1/4\lambda$	Negative controls
Replicate series	1:	+	+			
	2:	+	+			
	3:	+	+			
	4:	+	+			

The result in this example is the ideal one in which all four replicates give an end point at the labeled sensitivity of λ. The requirements are met provided that the geometric mean end point is between $1/2\lambda$ and 2λ. An implicit (but not explicit) requirement is that all replicates should clot at the 2λ concentration and none should clot at $1/4\lambda$. In addition, while not a requirement, it is reasonable to expect that the end points of any two replicate series should not differ by more than a factor of two.

For the photometric methods, at least three standard endotoxin concentrations are tested in at least triplicate, and the different standard concentrations should not differ by more than a factor of ten. A standard line is constructed and the absolute value of the correlation coefficient (lrl) must be at least 0.980. As was noted above in the discussion of regulatory requirements, for the photometric methods there is no check against manufacturer's criteria or other external standard, unlike the gel-clot method. Consequently, it is quite possible to make a dilution error and still meet the requirement for linearity. It is therefore recommended here, but not in any regulatory document, that other parameters such as OD values, onset time, and y intercept be routinely checked for atypical values (slope is not a good indicator of such errors).

CSE Potency Determination

The only endotoxin standard referred to in the pharmacopeial endotoxins test chapters is the primary standard endotoxin (such as the USP endotoxin RS). In practice, for most testing, a CSE that has been standardized against a primary standard is used. The quantity of CSE is typically expressed in units of mass. To convert the units of mass to endotoxin units (EU) of international units of endotoxin (IU), the potency of the CSE must be determined.

It is important to remember that a CSE potency is only applicable for the lot of LAL reagent and CSE with which it was determined. A potency determined with one reagent lot combination cannot be assumed to apply to any other combination. Consequently, only a CSE provided with a kit should be labeled in EU and the potency given on an accompanying certificate of analysis. The potency of this CSE should not be assumed to apply to other lots of LAL reagent. CSE provided for general use should be accompanied by a certificate of analysis specific to the lot of CSE and the particular lot of LAL reagent with which it will be used. Alternatively, the potency of the CSE can be determined by the user, again with a specific lot of LAL reagent.

Regardless of the test method, the potency of a CSE is determined by testing parallel series of dilutions of RSE and the CSE. The units are EU/mL for the RSE and units of mass (often ng/mL) for the CSE. The range of CSE concentrations to give equivalent activity to RSE has to be estimated. A good working assumption is 10 EU/ng for most CSEs, but it is wise to add an extra CSE concentration to the upper and lower ends of the series in case the potency of the CSE is higher or lower than this.

Determining CSE Potency by the Gel Clot Method

For the gel-clot method, a series of dilutions is prepared for both RSE (2λ, λ, $1/2\lambda$, and $1/4\lambda$) and CSE. If the test is being conducted to verify the CSE potency given on a certificate of analysis, that potency can be used to determine the appropriate concentrations of CSE to be tested. Divide the 2λ, λ, $1/2\lambda$, and $1/4\lambda$ concentrations by the stated potency to give the concentrations of CSE to be tested.

The dilutions of the two standards are tested in quadruplicate. From the end points of the four replicates series, the geometric mean end point endotoxin concentration is determined for

each standard, in EU/mL for the RSE and ng/mL for the CSE. The geometric mean of the RSE (which must be within a factor of two of the labeled sensitivity) is then divided by that of the CSE to give a potency of the CSE in EU/ng.

The procedure is described in Appendix B to the FDA Guideline, which is the BET from the first supplement to USP XXI and was current at the time the Guideline was issued in 1987. It calls for testing one vial of RSE and four vials of CSE. In a subsequent revision of the BET, but before the chapter was harmonized and reference to CSE was removed, the requirement was changed to a single vial of CSE.

The following is an example of a CSE potency determination using a LAL reagent with a sensitivity of 0.06 EU/mL:

RSE	2λ	λ	$1/2\lambda$	$1/4\lambda$	Negative controls
(EU/mL)	0.125	0.06	0.03	0.016	0
	+	+			
	+	+			
	+	+			
	+	+			

$$\text{GM RSE endpoint} = \text{antilog}\frac{4.89}{4} = 0.06\,\text{EU/mL}$$

CSE (ng/mL)	0.025	0.0125	0.006	0.003	0.0016	0.0008	Negative controls
	+	+	+				
	+	+	+				
	+	+	+				
	+	+	+				

$$\text{GM CSE endpoint} = \text{antilog}\frac{8.89}{4} = 0.006\,\text{ng/mL}$$

$$\text{CSE potency} = \frac{\text{GM RSE endpoint}}{\text{GM CSE endpoint}} = \frac{0.06\,\text{EU/mL}}{0.006\,\text{ng/mL}} = 10\,\text{EU/ng}$$

As is generally true for the gel-clot assay, the method does not have the resolution to determine potencies between twofold dilutions. If the "true" potency is 7 EU/ng, with some LAL reagents the potency may be determined to be 10 EU/ng, with others it could be 5 EU/ng. One reason for this is that reagent sensitivity is determined using a twofold series of endotoxin dilutions, so the labeled sensitivity is an approximation. This lack of precision of the method is one reason for the potency of a CSE determined using one lot of LAL reagent may differ from that obtained using another lot.

Determining CSE Potency by the Photometric Methods

The procedure for potency determination by photometric methods is given in Appendix C to the FDA Guideline. Slight variations are described for different test methodologies but the principles are the same. A series of RSE concentrations are prepared to enable construction of a standard curve. These are tested in parallel with four series of known concentrations of CSE (in units of weight, e.g., ng/mL) prepared from four vials of CSE. The CSE concentrations are treated as unknowns and their endotoxin concentrations determined against the RSE standard curve. For each measured CSE concentration that falls within the range of the RSE standard curve, the measured concentration (in EU/mL) is divided by the known concentrations (in ng/mL) to give a potency in EU/ng. Then the mean potency of the individual CSE concentrations is determined.

Test Method Development

Test method development is conducted to determine the interference characteristics of the product/sample and is sometimes called product characterization. The goal is to develop a method to overcome interference with the endotoxin/LAL reaction caused by the product and to determine whether the product contains contaminant endotoxin (or glucan) at a concentration that might interfere with the test for interfering factors, which is conducted to validate the test. If it is necessary to treat the sample in any way other than by dilution, it must be demonstrated that the selected treatment does not mask or remove endotoxin in the sample. Test method development is the foundation on which validation is built (where validation consists of successfully performing the USP BET test for interfering factors described in the next section).

As dilution is the simplest and most common means of overcoming interference, the first step in the method development process is to determine the MVD as has already been described. To determine the greatest possible MVD, the maximum possible test sensitivity should be used in the calculation, even though it may not be necessary (or desirable) to use this sensitivity.

The pH of the sample/LAL reaction mixture should be measured to assure that it is within the range specified in the package insert for the LAL reagent. It is the pH at the concentration/dilution at which the test is conducted that is important. If the MVD is 500 and the pH of the reaction mixture with the undiluted sample is out of range, but it is in range at a 10-fold (10×) dilution, then test the product at dilutions made from the 10× dilution. There is no need to adjust the pH of the undiluted sample or to use additional buffer. If necessary, pH can be adjusted with endotoxin-free NaOH or HCl. A sample with a pH outside this range does not always need adjustment. WFI tends to be slightly acidic, typically having a pH of about 5.8, but it is unbuffered and should not need adjustment prior to testing. The buffering capacity of the LAL reagent may be sufficient to bring the pH of the reaction mixture (sample plus LAL reagent) into range, and this should be verified by measurement. Alternatively, a LAL reagent reconstituted in a suitable buffer may be used.

The initial objective of preliminary testing for method development is to determine whether dilution alone is sufficient to overcome interference, without exceeding the MVD. The goal is to determine the dilution required to overcome interference (DROI). This dilution contains the highest noninterfering concentration of product (sometimes referred to as the NIC) and no significant endotoxin contamination. For the gel-clot method, the DROI is the least dilution at which the PPC (sample "spiked" with a known concentration of added endotoxin) clots but the sample does not. For photometric methods, it is the lowest dilution at which a valid PPC recovery is achieved (i.e., quantified within the range of 50% to 200% of the nominal concentration).

Regardless of methodology (gel-clot or a photometric), the usual procedure is to test a series of dilutions of product. The greatest dilution tested might be the MVD or it might be the last two dilutions might bracket the MVD. At each dilution, the product is tested both "unspiked" (no endotoxin added) and "spiked" with added endotoxin. The spike concentration is the same at each dilution, but the product concentration declines down the dilution series.

The appropriate endotoxin spike concentrations depend on the test methodology and are generally the same as those specified in the pharmacopeial endotoxins test chapters for the test for interfering factors. For the gel-clot method the final concentration of added endotoxin is 2λ (which is double the label claim sensitivity). This is the lowest endotoxin concentration that should always clot. For the photometric methods, the endotoxin concentration of PPCs should be equal to or near the middle concentration of the standard curve.

Spiking Methods

There are three different methods of achieving the desired endotoxin spike concentration. First is the addition of a small volume (≤10% of the sample volume) of concentrated endotoxin to sample to give the desired final concentration. The volume added is small enough so that it does not dilute the sample significantly. For example, to achieve a final endotoxin concentration of 0.125 EU/mL, 0.01 mL (10 µL) of 1.25 EU/mL endotoxin

standard can be added to 0.1 mL of sample in the reaction tube. This is sometimes referred to as the hot spike method. It is the only way to spike an undiluted sample without significantly diluting it.

For the second method, a volume of standard endotoxin at double the desired final spike concentration is mixed with an equal volume of sample at double the desired final concentration. The endotoxin solution and the sample dilute each other to the final desired concentrations. The disadvantage of this method is that it cannot be used to test undiluted sample; the minimum dilution is a twofold. The advantage of the method is that the same endotoxin dilutions can be used to prepare endotoxin standards and to prepare spiked product.

The third method is for preparing a series of dilutions of sample spiked at particular endotoxin concentration and is used after preparing an initial volume of spiked sample by one of the first two methods. The spiked sample is then diluted with diluent (usually LRW) containing endotoxin at the spike concentration. The product concentration is reduced at each dilution but the endotoxin concentration remains constant. This procedure is ideally suited to preliminary testing for method development.

In the gel-clot test, the unspiked sample should not clot. If it does, it either contains contaminant endotoxin (the most likely possibility), or there is an interference that causes a false positive such as (1→3)-β-D-glucan (unless an endotoxin specific test is being run) or a serine protease that mimics the activated clotting enzyme and cleaves the clotting protein, coagulogen. The spiked sample should clot. If it does not, the sample is inhibiting the clotting reaction. The +/ twofold error in the gel-clot test may "hide" interference. PPCs may still clot in the event of 50% inhibition. This is one of several reasons not to validate the test at the DROI. It should be noted that, in the case of the gel-clot method, this test is only capable of detecting inhibition, not enhancement.

For photometric methods, in order for the test to be valid the measured endotoxin concentration of PPCs should be within 50% and 200% of the known concentration and ideally well within that range. Spike recoveries are determined relative to the nominal (known or theoretical) concentration of the PPC. However, it is often useful to compare recovery with the measured concentration of the equivalent standard (or to a positive control consisting of water spiked in the same way as the PPC if PPCs and standards were not prepared in the same way). Greater or lesser spike recoveries indicate enhancement or inhibition, respectively.

It is not always possible to overcome interference by dilution of the sample, even after increasing the test sensitivity, which increases the MVD and the scope for dilution. In this case one strategy is to try a different test method. The formulation of the reagent for another method may be better suited to the chemistry of the product and enable interference to be overcome at a product dilution less than the MVD. Or, changing to a more sensitive method will result in a greater MVD, and interference may be overcome at a greater dilution.

For some samples, neither dilution nor a change of test method is sufficient to overcome interference and some type of treatment is required. The goal of treatment is to eliminate the interference without removing any endotoxin that might be present in the sample. When a treatment other than dilution is used or when buffers are used in a manner not described in the LAL reagent manufacturer's instructions, it is necessary to validate the treatment to demonstrate that it removes interference but not endotoxin. Use of a LAL reagent manufacturer's buffer in accordance with the package insert does not constitute a "treatment" requiring special validation. To validate a treatment, the BET states "... perform the assay ... described above using the preparation to be examined to which USP Endotoxin RS has been added and which has then been subjected to the selected treatment," where the "assay described above" is the test for interfering factors. This means that standard endotoxin must be added to the sample prior to treating it. An appropriate addition would result in a concentration in the product of twice the endotoxin limit. The addition should either be a small volume of concentrated endotoxin that will not significantly dilute the product or the dilution should be taken into account. The "spiked" product should be subjected to the selected treatment and then tested. The pharmacopeia do not state what an acceptable recovery is but a recovery between 50% and 200% is reasonable and is consistent with the recovery specified for PPCs.

One option for addressing interference is described for photometric methods in the FDA Guideline. This is the use of a "product standard curve." This involves preparing the standard endotoxin concentrations in the product to be tested (or in a dilution of product). The advantage is that any degree of interference in the sample is also experienced by the standard endotoxin concentrations. The product (or dilution) cannot contain a significant amount of endotoxin (i.e., the endotoxin concentration must be less than that of the lowest standard endotoxin concentration), otherwise the standard curve will be distorted at the lower concentrations. Product standard curves should be considered a method of last resort because of the influence of contaminant endotoxin on the standard line and the possibility of different degrees of interference between lots of product.

From the results of the preliminary characterization tests performed for method development, a product dilution/concentration is selected for validation (the test for interfering factors) and subsequent routine testing. The criteria for this selection process are as follows:

- The product dilution should contain significantly less endotoxin than the endotoxin limit.
- The product dilution selected for validation should be at least one- to twofold greater than DROI (the first dilution in which no interference is evident).
- The dilution selected must not exceed the MVD and, if possible, should be less than the MVD.

The method development process should be documented, including the rationale for decisions made. This documentation can then be referenced to explain the dilution selected for the test for interfering factors and the need for any sample treatments.

The Test for Interfering Factors

Provided that the method development process has been properly executed, performing the test for interfering factors should be straightforward. However, it is important that it be done correctly; if it is not, interferences may occur during the test for interfering factors or subsequent routine testing of the product. The purpose of the test is to demonstrate that the ability to detect endotoxin in a particular product or sample type is unaffected by the presence of the product. To have confidence in the test, it must be clear that negative test results are due to the absence of endotoxin, not to inhibition of the reaction that is preventing detection of endotoxin in the sample.

To validate a test method for a particular product, the FDA Guideline states that the test for interfering factors should be performed on three separate lots of product. The test is conducted at single dilution (i.e., a single concentration) of product. Upon successful completion, the method is considered validated for the product dilution/concentration at which it was conducted and also at greater dilutions. Testing should not be performed at a lesser dilution (higher concentration) without revalidation. The test should be validated for each sample type, regardless of whether the sample is finished drug product, medical device extract, in-process sample, component, raw material, WFI, or any other type of sample.

The number of units tested per lot is not specified in any of the regulatory documents. Drawing from the recommendations for routine testing of drug products in the FDA Guideline, it is reasonable to test a minimum of three units taken from the beginning, middle, and end of the lot. It is also reasonable to assume that these units may be pooled for testing. For medical devices, the number of extracts and their handling (e.g., pooling) should be the same as is planned for routine testing.

Gel Clot Method

For the gel-clot method, parallel label claim verifications are performed in water and in product (or product dilution). One series of standard endotoxin concentrations is prepared by diluting with water; the second series is prepared with the same endotoxin concentrations diluted in the selected concentration of product. The standards in water (the control series) are

tested in duplicate and the series of standards in product are tested in quadruplicate. Duplicate negative controls are included with quadruplicate product negative controls consisting of unspiked product at the selected test dilution.

For both the series of standards in water and the series in product, the geometric mean end point must be within twofold of the labeled sensitivity of the LAL reagent. There is no requirement regarding agreement of the end points of the two series with each other. Prior to harmonization of the USP, EP, and JP endotoxins test chapters, the EP required that the geometric mean end points be within a factor of two of each other, and this is a reasonable goal, but it is not a requirement. End points differing by more than a factor of two in parallel tests suggest that there might be interference that needs investigation.

Example of the test for interfering factors by the gel clot method:

Assume a label claim sensitivity (λ) of 0.125 EU/mL and a product with an MVD of 100. (The MVD could be increased to 400 by switching to a LAL sensitivity of 0.03 EU/mL.) Also assume that preliminary testing indicated inhibition at dilutions down to 1:4, and that this product is to be validated at a 1:25 dilution.

Standards in water (EU/mL):

0.25	0.125	0.06	0.03	Negative Controls
+	+			
+	+			

Geometric mean end point 0.125 EU/mL.

Standards in sample (EU/mL) at a 1/25 dilution:

0.25	0.125	0.06	0.03	Sample negative control
+	+			
+	+			
+	+	+		
+	+			

Geometric mean end point 0.11 EU/mL

The geometric mean end points for both the series of standards in water and in product are within twofold of the labeled sensitivity of the LAL reagent. (Also, the end points are within a twofold of each other.)

Photometric Methods

For the photometric methods, just as with the gel-clot method, the sample of product is tested at a dilution not to exceed the MVD (usually as determined during method development). Like the gel-clot method, the sample is tested unspiked and spiked with added endotoxin. However, unlike the gel-clot method, which requires that the sample be tested in quadruplicate, the test is conduct in duplicate. According to the pharmacopeial endotoxins test chapters, the concentration of endotoxin in the PPC should equal one of the concentrations from or near the middle of the standard curve. (There are different recommendations for PPC concentrations in the 1987 FDA Guideline and the 1991 Guidance, depending on the specific test method, but these have been superseded by the pharmacopeial chapters.)

In order for the test to be valid, the harmonized pharmacopeial endotoxins test chapters require that the negative control must contain less endotoxin than the lowest standard concentration, λ. (The negative controls should actually contain significantly less endotoxin than the lowest standard because concentrations of contaminant endotoxin approaching λ distort the standard curve.) The mean measured concentration of added endotoxin in the spiked product is required to be within 50% and 200% of the known or nominal concentration. If any endotoxin is detected in the unspiked product, this is subtracted from the total measured in the spiked product prior to determining the percentage recovery of the added endotoxin.

Although the endotoxins test chapters allow for a recovery range of 50% and 200%, it is recommended that recoveries closer to 100% be expected. If recoveries near the extremes of the range are accepted for the test for interfering factors, it is quite possible that a subsequent batch of product may have slightly different interference characteristics and cause the PPC to fall outside the allowed range, resulting in an invalid test result.

Routine Testing

Once the BET has been validated for a particular product, routine testing may be conducted. For finished product, every lot of product should be tested. (The AAMI/ANSI Standard, ST72, "Bacterial Endotoxins Test Methodologies, Routine monitoring, and Alternatives to Batch Testing" does make provision for not testing every batch of finished product. This document is included in the recognized standards listed by the FDA Center for Devices and Radiological Health. The scope statement limits the standard to medical devices but notes that the requirements and guidance given may be applicable to other health care products.)

Regardless of test method, all routine tests should be conducted in at least duplicate; negative controls must be included with all tests, and standards or appropriate positive controls must also be included.

Routine Testing by the Gel Clot Method

As previously stated, the FDA Guideline states that a standard series need not be included with every gel-clot test once consistency of standard end points has been demonstrated in the test laboratory. In that case, a standard series should be run for at least the first test of the day. If the lysate lot, the CSE lot or the test conditions change, a new standard series should be run for the new conditions. For the limits test described in the BET, the required controls are negative controls (LRW) and positive controls (endotoxin diluted in water) at a concentration of 2λ. However, to meet the FDA Guideline recommendations, a full series of standards should be included with the first test of the day. The sample is tested at the validated dilution with and without added endotoxin at a final concentration of 2λ, the latter being the PPC.

In order for the test to be valid, the negative controls must not clot and both the positive control (in water) and the PPC (in product) must clot. If these conditions are met and the sample tests negative, it meets the requirements of the test. If the sample tests positive at a dilution less than the MVD, it may be diluted not to exceed the MVD and retested.

Example of the results of a gel-clot limits test:

Negative controls	2λ positive controls
	+
	+
Sample (unspiked)	PPC (2λ)
	+
	+

In this test, all controls meet specification indicating that the test is valid. The sample tests negative and therefore meets the requirements of the test.

For the gel-clot assay described in the pharmacopeia, in addition to negative controls, a full series of standards (at concentrations of 2λ, λ, $1/2\lambda$, $1/4\lambda$) is specified. (This is in contrast to the Guideline allowance for only testing a full series of standards with the first test of the day. In the absence of a full standard series, a 2λ positive control is included.) A series of dilutions of the sample, not exceeding the MVD, is tested. While not stated explicitly, the first dilution should be that at which the test was validated. Also, a PPC at a concentration of 2λ is included for the first dilution of the sample.

For an assay to be valid: the negative controls must be negative, the geometric mean end point of the series of standards must confirm labeled sensitivity of the LAL reagent within a factor of two, and the PPC must test positive. If the geometric mean of the endotoxin concentration in the sample is less than the limit stated in the monograph in a valid test, the sample meets the requirements.

The limits test is typically used for routine testing of product. The assay procedure is used to quantify the endotoxin concentration in a sample and might only be run if the sample tested positive in the limits test. The assay would serve to determine whether the sample meets the endotoxin limit specification at a dilution no greater than the MVD. In this case the assay should be designed so that the series of dilutions tested includes the MVD. If necessary, the assay can be run using dilutions beyond the MVD for informational purposes to determine the endotoxin concentration of a sample that fails to meet the endotoxin specification (though this is beyond the scope of the pharmacopeial endotoxins test chapters).

Routine Testing by the Photometric Methods

The procedure in the pharmacopeia for routine testing by the photometric methods is the same as that used for the test for interfering factors described above. There are other procedures in the FDA Guidance documents, but as these precede the current BET, and as they are less straightforward than the BET, adherence to the BET is recommended.

As was described in the discussion of the Guideline and/or the Guidance above, these documents provide for use of an archived (stored) standard curve for routine testing if consistency has been demonstrated in the user's laboratory. The standard curve may be omitted provided a standard control (a spike in water at the same concentration as the PPC) is included. Endotoxin concentrations are then determined relative to an archived standard curve. In order for the test to be valid, the determined value of the standard control should be within 25% or the nominal concentration. Unless it can be demonstrated that standard controls can be routinely detected within 25% using the archived curve, this approach is not recommended. However, archived curves have been adopted in commercially available readers in which the LAL reagent is presented in cartridges. Meeting the recommendation for inclusion of standard controls may not be possible if the system capacity is limited.

For drug products, the Guideline calls for testing at least three samples from each lot of product. The samples should represent the beginning, middle, and end of the production run. The Guideline allows for samples to be pooled for testing. However, if samples are pooled they should not be tested at the MVD. This is in case one vial is contaminated but the others are not and endotoxin in the contaminated vial is diluted below the limit by the clean product when the samples are pooled. The MVD should be reduced by a factor equal to the number of vials pooled. This means that the endotoxin limit for the pooled samples is reduced by the same factor. This is not written in any regulation, but it is necessary to assure that all of the units tested meet specification and has been stated in public meetings by US FDA officials.

RETESTING

Both the pharmacopeial endotoxins test chapters and the FDA Guideline include provisions for retesting samples that contain endotoxin at or above the endotoxin limit. A test result in which the endotoxin concentration of the sample fails to meet the specification for the product is an OOS result. Before considering procedures for retesting, it is important to note that an invalid test is not a test failure, that is, it is not an OOS result. If the requirements for the controls are not met, the test should be invalidated and the reason for invalidating the test should be documented. The test may be repeated. A clear distinction should be made between repeating a test because the initial test was invalid because controls did not meet the requirements for a valid test and repeat testing following an OOS result in a valid test. A product cannot be said to fail to meet specification as a result of an invalid test (though the result might indicate that it would likely fail to meet specification), and product cannot be released on the basis of an invalid test. It should be noted that for the gel-clot method, a product has not failed to meet specification until it tests positive in a valid test conducted at the MVD. Performing an assay after a positive result in a limits test at a dilution less than the MVD does not constitute a retest; it is additional testing to determine whether the sample meets the endotoxin specification.

The USP and EP specify a retest of the gel-clot limits test if one of the two replicates clots and the other does not. The USP medical devices chapter (44) permits a retest of medical devices that fail to meet specification. There is provision for two retests in the FDA Guideline, which requires the inclusion of standards "when confirming end-product contamination," that is, when retesting. The first retest is to ensure that the test itself was not contaminated and is a repeat of the original test of the sample in question (pooled or not) but with double the original number of replicates. The second retest only applies to pooled samples and is to check for the possibility that the samples of the article were contaminated after they have been taken and pooled. For the second retest of 10 new units of the product tested individually (not pooled) and all must pass the test.

The US FDA has issued a Guidance document (46) on OOS results, which provides the context in which retests of samples giving OOS results should be performed. One of the essential elements of this document is that retests of OOS results should not be conducted before first conducting an investigation. The investigation should be conducted in accordance with an approved OOS SOP, and justification for the retest should be documented. A distinction should be drawn between testing for investigational purposes and a repeat of a release test. As was stated above, the distinction between invalid tests (controls not all valid) and OOS results for product should be clear. One concern that must be addressed is that if a product fails an initial test, why is the repeat test any more valid than the initial test? Unless there is a reason to invalidate the initial test, both the initial OOS result and any subsequent results should be taken into account by the quality unit (typically quality assurance) when the decision whether to release a batch of product is being made. Finally, the guidance addresses the issue of averaging; caution should be exercised regarding "averaging into compliance." This is not a concern if all replicates contain less than the endotoxin limit concentration (as they must for the gel-clot method).

MATERIALS AND IN-PROCESS TESTING

For materials (including excipients) and in-process samples, the same principles apply as for release testing of finished products. Preliminary testing should be conducted to develop an appropriate test method, and the test should be validated by performing the test for interfering factors. A major difference between testing finished products and excipients, materials, and in-process samples concerns endotoxin limits. For finished products, limits are clearly specified in product monographs; for excipients, materials, in-process samples, and API, appropriate limits have to be set by the manufacturer.

Raw materials should first be assessed for the need for an endotoxin specification. This can be done by compiling a list of materials and supplies and then identifying those for which an endotoxin specification is clearly not required. For example, there is no need for an endotoxin limit for glass vials that will be depyrogenated using a validated process. There is a very low risk that the vials will contribute endotoxin to the finished product. Materials that may require an endotoxin specification can then be assessed on the basis of the risk that they might be contaminated and contribute endotoxin to the finished product.

When setting endotoxin limits for excipients and other components, it should be noted that the risk of endotoxin contamination is much greater for some materials than for others. Materials derived from natural (plant and animal) sources are more likely to be contaminated with endotoxin. Other materials may contain little or no endotoxin because of their origin or manufacturing procedure. Inorganic salts typically have a low level of contamination. Some materials may be depyrogenated prior to incorporation in the formulation.

It was noted above that endotoxin limits in pharmacopeial monographs (and those calculated from the dose of API) are stated per unit of API, but that the stated limit applies to the whole finished dosage form. If an in-process endotoxin limit for the API is set at the limit stated in the monograph, there is no allowance for the occurrence of endotoxin in the excipients, containers, and closures, etc. Appropriate in-process limits should be set for the API and other components of the product. It is important *not* to calculate the endotoxin limit for each component as if it were a separate product. This would allow each component to contain

passing amounts of endotoxin based on individual doses, but the cumulative total in the formulated product could exceed the endotoxin limit for the product.

The logical approach to determining endotoxin limits for excipients is to work back from the endotoxin limit for the finished product, which can be calculated as previously described on the basis of the dose of the API. Then the decision can be made as to whether it is necessary to apply a "safety factor," such as dividing the limit by two. The endotoxin limit must then be divided between the various components of the product. A simple and unbiased approach is to assign endotoxin limits on the basis of mass. This is accomplished by calculating the total mass of all of the components in the product (including the API and all the excipients) in a given unit of product, such as one milliliter. Then the limit per unit of product (e.g., EU/mL) is divided by the total mass per unit (e.g., mg/mL). This gives a limit per unit mass (e.g., EU/mg) that applies to all of the components, not just the API.

A more sophisticated approach applies knowledge of the nature and origin of the components and takes account of any historical data from past endotoxin testing. This information is used to modify the limit calculated on the basis of mass and set limits for the individual components. Thus, more stringent limits can be set for components that are unlikely to be contaminated with endotoxin, allowing higher limits to be set for those that are likely to be contaminated, notably those of natural origin.

To put limits calculated for an individual excipient into a larger context, the limit can be determined for each of the products of which it is a constituent. The most stringent of the limits can then be selected as the limit for that material, allowing it to be used in any of the product of which it is a component. At the time of writing, the USP is in the process of developing endotoxin limits for excipients. When such limits are published, they should be used as an upper limit. If the limit determined as described above for a specific products result in a more stringent value, then the more stringent limit should be adopted.

DEPYROGENATION AND ITS VALIDATION

Depyrogenation is the removal or destruction of pyrogens (and particularly endotoxin) from an article. Endotoxin is not only the most significant pyrogen in most situations, it is also the most refractory to degradation. Consequently, conditions required to destroy or remove endotoxin will also destroy/remove the great majority of other pyrogens, so the term depyrogenation is not inappropriate, even though it is generally endotoxin removal that is of concern. Depyrogenation processes should be validated to show a minimum level of endotoxin removal or destruction. The degree of removal/destruction is typically expressed as a multilog reduction from an initial concentration.

Depyrogenation can be accomplished in a number of ways with thermal destruction of endotoxin by dry heat being the most common. Other methods of destruction may also be effective, or an endotoxin removal process may be used. The latter is best illustrated by rubber stoppers as they are not suitable candidates for dry heat treatment. In the discussion that follows, depyrogenation by endotoxin removal is considered first, followed by destruction of endotoxin.

Methods of Depyrogenation

Depyrogenation by Removal of Endotoxin

Distillation. This removes the solvent, typically water, from the endotoxin and other impurities and is very effective (49). It is possible to overwhelm a still if the endotoxin load or "pyroburden" is too high.

Reverse osmosis. In the depyrogenation of water by reverse osmosis (RO), water is forced through very small pores in a membrane against the osmotic gradient; water passes through the membrane but endotoxin does not. The molecular weight cutoff (MWCO) for RO membranes is generally not specified as it is for ultrafilters. However, as an RO membrane is generally one that will remove salts (50) and as the molecular weight of sodium chloride is 58.44, the size exclusion of RO membranes is clearly less than 100 Da.

Ultrafiltration. Ultrafiltration is an effective process scale treatment that relies on the solution or molecules to be depyrogenated being significantly smaller than endotoxin (51,52). A membrane rated to retain molecules (or aggregations thereof) of 100,000 Da will generally remove endotoxin from aqueous solutions because the LPS is aggregated. However, if the physicochemical conditions of the solution cause the LPS to disaggregate, a lower MWCO ultrafilter is needed. A membrane with a 10,000 Da MWCO can be expected to remove endotoxin from most solutions.

Ion exchange resins. An anion exchange resin will remove the negatively charged endotoxin. Use of this technology to remove endotoxin and purify an enzyme has been described by Belanich et al. (53). In common with activated carbon (discussed in the following text), ion exchange resin is best suited to batch processing. Unless carefully sanitized, an ion exchange resin can become fouled by bacteria and might contribute endotoxin to the system rather than remove it. This is true in water systems as well as other applications.

Activated carbon. Activated carbon binds and removes organic molecules, including endotoxin, and can be effective in the depyrogenation of solutions (54). Activated carbon can be depyrogenated by dry heat prior to use for depyrogenation. It can be added to the solution to be depyrogenated or the solution can be passed through a column or cartridge containing activated carbon. As is the case for ion exchange resins, it is better suited to batch processes but it readily becomes fouled and can become a source of endotoxin if resident in a system for an extended period. The effectiveness of activated carbon is reportedly increased when combined with autoclaving 121°C for 90 minutes (55). Activated carbon can be removed by settling, filtration, or centrifugation, or by a combination of these approaches. For injectable products or their components, care must be taken to assure removal of particulates.

Charge modified media. Positively charged filter media can remove endotoxin from aqueous and salt solutions, though may not be effective for protein solutions. Historically asbestos was used in this application (56), but this is now specifically prohibited for pharmaceuticals in the revisions to cGMPs for pharmaceuticals (21 CFR Part 211.72) that became effective on December 8, 2008. Filters with a positive zeta potential (i.e., a positive charge), commonly nylon, are now used (e.g., Blanden et al., 1991) (57).

Affinity devices. Devices with a specific affinity for endotoxin are available for endotoxin removal. These include Polymyxin B on sepharose or agarose chromatography columns, heparin affinity devices, and histamine sepharose. These may not be effective in certain protein solutions, and it may be necessary to adjust salt concentration and pH to obtain maximum selective binding of endotoxin.

Washing/rinsing. Washing or rinsing can used to remove endotoxin from solid articles that cannot be depyrogenated by dry heat. This may be a hot WFI rinse or may involve use of a chemical agent followed by rinsing. Berzofsky et al. discuss depyrogenation of rubber stoppers (58); Feldsine et al. (59) and Berman et al. (60) address rinsing of containers. Chemical agents to aid the process include surfactants, NaOH (typically in the range of 0.05 0.5 M), or commercial cleaning agents.

Depyrogenation by Chemical Destruction of Endotoxin

Acid hydrolysis. Mild acid hydrolysis severs the ketosidic linkage between the lipid A and the sugar moiety of the structure (61). The free lipid A then aggregates and is relatively nonpyrogenic until it is solubilized. Further hydrolysis of the lipid A by acid may truly reduce the pyrogenicity. Reported acid treatments include 0.12 M HCl for 30 minutes followed by extensive rinsing (62); 0.05 M HCl for 30 minutes at 100°C (63); 1% glacial acetic acid for 2 to 3 hours at 100°C (61).

Base hydrolysis. Treatment with alkaline solution is used in cleaning and treatment to reduce endotoxin contamination, primarily of equipment such as tanks and tubing. The mechanism of

depyrogenation is by saponification of the fatty acids of lipid A. Sodium hydroxide is commonly used at concentrations between 0.05 and 0.5 M. In addition to destruction of endotoxin by hydrolysis, at high pH the surface affinity of endotoxin is reduced and solubilization is increased, allowing it to be rinsed away. In many instances, particularly if elevated temperatures are not used, this is probably as important as destruction.

Sodium hydroxide requires substantial amounts of water (usually WFI or other water of low endotoxin concentration) to rinse it away (but it is easy to determine when the residue has been removed by monitoring pH of the water). Rinsing with copious volumes of water will aid depyrogenation. KOH is also effective and is more readily removed by rinsing.

The rate of depyrogenation by alkaline hydrolysis is increased by even moderate heating. For example, treatment with 0.25 M NaOH at 56°C for 1 hour has been shown to be effective (64). Depyrogenation by 0.1 M NaOH is also reportedly enhanced in the presence of 95% ethanol or 80% dimethylsulfoxide (65).

Oxidation. Hydrogen peroxide has been shown to be an effective agent for depyrogenation at high concentrations at elevated temperatures (27% at 100°C). It showed some effectiveness at 2.7% and 37°C (66), and it has been suggested that it is probably more effective at higher pH. Ozone has been used to produce water of low endotoxin concentration (67), and effectiveness is reportedly increased in the presence of ultraviolet light (68). Sodium hypochlorite (bleach), an oxidizing agent commonly used in sanitization and cleaning, is not generally recognized as being effective in depyrogenation.

EtO is another oxidizing agent. It is widely used to sterilize material, particularly medical devices. EtO has been reported to inactivate endotoxin but it is not sufficiently effective to achieve a greater than 3 log reduction (69,70). However, it should be noted that it is inhibitory in LAL tests (71), and articles must be allowed to outgas before being tested for endotoxins.

Depyrogenation by Physical Destruction of Endotoxin

Radiation. Sterilizing doses of radiation (γ-radiation) are not sufficient to significantly reduce endotoxin concentration (70). High doses will destroy endotoxin but may affect articles being treated, particularly plastics.

Moist heat. Autoclaving is not generally regarded as an effective means of depyrogenation, but it does have some effect. Long cycles, especially at elevated temperature and pressure, will destroy endotoxin, for example, 5 hours at 20 p.s.i. and pH 8.2, or 2 hours at pH 3.8 (72). At 15 p.s.i. (the pressure typically used in autoclaves), significant reduction in endotoxin concentration has been reported after three hours (55). As stated above, the combination of autoclaving and activated carbon has been found effective. Also, Bamba has demonstrated endotoxin destruction by autoclaving in the presence of a nonionic surfactant as well as some level of depyrogenation by autoclaving alone (73).

Dry heat. Dry heat is the most effective and commonly used method for depyrogenating many articles. Provided that the articles can tolerate the heat exposure, it is the method of choice. It is widely used for glass and stainless steel and can also be used for Teflon®. Silicone (e.g., tubing) can be depyrogenated by dry heat, but temperatures of 250°C render tubing brittle and prone to cracking and breakage (unpublished observation).

Temperatures in excess of 180°C effectively destroy endotoxin (74,75) with the time required decreasing as temperature increases. When discussing depyrogenation, the USP BET states "Commonly used time and temperature settings are 30 minutes at 250°" and references the informational chapter <1211> (43) "Sterilization and Sterility Assurance of Compendial Articles."

Some terminology, which has been adopted from theory of sterilization, has been applied to depyrogenation. The model for sterilization is one of log linear kinetics of lethality over time under a given set of sterilizing conditions. This is illustrated by the D value, which is the time required to give a 1 log (90%) reduction in the number of viable organisms at a given temperature/pressure thus a time of $3 \times D$ will give a 3 log reduction. For depyrogenation,

the D value is the time required for a 1 log destruction of endotoxin at a given temperature. The z value is the temperature change required to change the D value by 1 log. It requires D value to be known at two temperatures and assumes linear destruction kinetics. The F value is the time required to give equivalent lethality (or destruction of endotoxin) at different temperatures; the time depends on the degree of lethality or destruction specified (2 logs or 3 log, etc.).

Unfortunately, destruction of endotoxin by dry heat does not follow log linear kinetics like sterilization (74,75). Consequently, the value of D, z, and F in discussions of depyrogenation is limited. This may explain the lack of agreement in the literature for the times at a given temperature required for given destruction of endotoxin (F values). Avis et al. (76) compared other depyrogenation studies with their own and cite F values of 10, 500, and 130 minutes for equivalent destruction of endotoxin at 250°C in three different studies.

While this may appear to complicate the selection of a time and temperature regime to be used for a dry heat depyrogenation process, at least 30 minutes at a temperature of at least 250°C is commonly used. This is well supported by the work of Hecker et al. (75), and these are the conditions cited in the pharmacopeial endotoxins test chapters.

Validation of Dry Heat Depyrogenation

Prior to starting the validation of a depyrogenation process, installation qualification (IQ) of the depyrogenation oven should be conducted. This should be followed by operational qualification (OQ) of the oven in which the heat distribution of the empty oven is mapped using temperature probes (typically thermocouples). All stages of the qualification process should be conducted according to preapproved validation protocols or procedures. The aim of the OQ is to ensure that temperature is suitably uniform in the oven and that there are no cold spots that may indicate either poor design (such as poor air circulation within the oven) or a defect, such as a leaking door seal. The time for the oven to reach operational temperature should be noted during this phase. This is useful as a reference and for comparison in future qualifications to determine whether oven performance has changed. Ideally the oven timer will not start until a specified temperature set point has been reached.

Once a functional oven is qualified and available, it may be necessary to conduct experimental work to determine the heating times of the various oven loads to be processed. Then, for performance qualification (PQ) of the oven, the heat distribution of each specific oven load configuration should be mapped with temperature probes in vials, vessels, or materials throughout the oven, including the middle of the load, to assure temperature uniformity and to identify the last point in each load to reach the minimum process temperature and the time taken for that to occur. The load configuration should be documented using diagrams and/or photographs. The lag time between the point at which the oven controller reaches temperature and the time at which the last point of the load reaches temperature should be identified and added to the cycle time. The lag time can be reduced somewhat by setting the oven temperature higher than the selected process temperature, such as 260°C for a process temperature of 250°C.

In addition, it must be demonstrated that at least a 3 log reduction in endotoxin is achieved throughout the oven load. This is done by placing vessels containing endotoxin in the oven and then assaying for endotoxin after they have been exposed to the depyrogenation cycle.

Endotoxin challenge articles should contain or carry at least 1000 EU endotoxin (per USP General Information chapter <1211> (43)), and it is recommended that this be interpreted to mean recoverable 1000 EU. Thus, if 20% is the lowest acceptable recovery, an addition of 5000 EU is appropriate. A new monograph titled "Endotoxin Indicator for Depyrogenation" (77) has been added USP 33. Challenge articles (endotoxin indicators) may be vials of endotoxin purchased for the purpose of performing depyrogenation studies. Alternatively, challenge articles may be prepared by adding a small volume of high-potency endotoxin to the article and then drying the added endotoxin on the articles by air drying or lyophilization (the former is more common and perfectly acceptable) (78). The recovery of added endotoxin from the challenge articles is then tested. Ideally, at least 20% of the added endotoxin should be recovered, but there is no specification for recovery.

Regardless of whether commercially prepared vials or indicators prepared in-house are used, the challenge articles are distributed throughout the oven, including the cold spot, in the load configuration being validated. Five vials per shelf in an X pattern, with a minimum of three shelves, top, middle and bottom, is a common arrangement, except in the smallest ovens. A number of articles/vials are left out of the oven to serve as untreated controls. The depyrogenation cycle is then executed and challenge articles are recovered for testing.

To extract endotoxin (or any remaining endotoxin) from the untreated and heat treated articles, a minimal volume of LRW is added to the article (or if the article is not a vessel, it must be extracted like a medical device). A small volume of water is used to minimize dilution of any endotoxin and maximize the chance of detecting it. The endotoxin concentration in the extracts is then assayed. The test method used must be capable of detecting at least a 3 log (1000-fold) reduction from the measured endotoxin concentration of the untreated articles. The endotoxin detected in the extract of the treated (or processed) article should be at least 3 logs less than that in the extract from the untreated controls. A 4 log reduction is desirable (but not required) as it will provide a high level of assurance that depyrogenation is effective.

The standard operating procedure for depyrogenation should state the conditions that will necessitate revalidation. Verification that the validated conditions are being maintained by the equipment and process should be conducted at least annually. If the physical data (temperature and time) assure that specified minimum conditions are met or exceeded, and show that the rate of heating is not significantly different from the previous validation, it may not be considered necessary to repeat the endotoxin destruction studies every year. Temperature probes and chart recorders must be calibrated regularly and not used when out of calibration. The specified, validated loading condition must not be exceeded and each individual oven should be validated.

CONCLUSION

Endotoxin is highly biologically active and ubiquitous in the natural environment. Living organisms have evolved effective defenses against exposure to it. Parenteral products bypass the protective barriers of the skin and the gut wall and have the potential to introduce endotoxin into the body where it can elicit a wide range of deleterious effects. Consequently, parenteral products are manufactured in a manner that controls and minimizes endotoxin contamination. Because of the risk of contamination and the severity of the effects of endotoxin, parenteral products must be tested according to a validated procedure, and they must meet endotoxin specifications to ensure that they are safe to use. This chapter provides an overview of the nature of endotoxin and its effects on biological systems, of the regulatory requirements that apply to parenteral products, and of some practical considerations regarding testing for and removal or destruction of endotoxin. An understanding of these issues will help ensure that the potential for endotoxin contamination is recognized and will help to identify and eliminate contamination when it is identified. Similarly, an understanding of how endotoxin interacts with different types of sample under various physicochemical conditions can assist in overcoming interference in endotoxin testing and ensure that rugged test methods are developed and employed. A goal in manufacturing of parenteral products is to ensure that endotoxin is kept out of the process, removed from it, or is maintained at subcritical levels so that the final release test of product is almost a formality.

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