Teaching Editorial

Sterility and Pyrogen Testing of Radiopharmaceuticals

Technologists, physicians, pharmacists, and other nuclear medicine personnel spend a significant part of every day being concerned about the quality assurance (QA) of various study components, especially the radiopharmaceuticals and the equipment employed. For radiopharmaceuticals, it is desirable to demonstrate that the agent is radiochemically pure, that the appropriate dosage has been drawn and assayed in a properly calibrated radioisotope dose calibrator, and that the pharmaceutical has been administered to the patient by the prescribed route of administration. Most of these tasks are performed by nuclear medicine staff members in accordance with existing rules and regulations affecting the practice of medicine and pharmacy with oversight by the Food and Drug Administration (FDA) and other government agencies.

The primary responsibility of the FDA is to insure that all drugs (including radiopharmaceuticals) are safe and efficacious. Of particular interest from the standpoint of safety is the assurance that parenteral drugs are sterile and apyrogenic. While the assessment of these parameters is generally the responsibility of drug manufacturers, and these tests are neither required nor routinely performed in nuclear medicine departments, it is expected that nuclear medicine staff be familiar with them. This is especially the case in departments with active Investigational New Drug (IND) protocols, which typically require sterility and pyrogen testing. Accordingly, the purpose of this editorial is to review the state-of-the art of sterility and pyrogen testing, including test methodologies, limitations, and requirements for performance of these tests.

According to FDA regulations, all pharmaceuticals produced for parenteral use in humans, radioactive or otherwise, must be certified sterile and pyrogen-free in accordance with test procedures outlined in the United States Pharmacopoeia (USP XXI) (*1*). The reasons are obvious when one examines the definitions of sterility and pyrogenicity. Sterility denotes absence of viable microorganisms that are potentially pathogenic and may cause disease. Apyrogenicity, on the other hand, denotes the absence of endotoxin, usually described as the degradation products of gram-negative bacterial cell walls.

Endotoxins are heat-stable, filterable, soluble compounds which produce symptoms of fever including chills, joint pain, and headaches that occur following intravenous or intrathecal administration of materials contaminated with these substances. Although pyrogenic reactions usually are mild and short-lived, some are quite serious. Endotoxin shock, if not treated rapidly, can cause respiratory and circulatory collapse, resulting in death.

From the standpoint of definitions, it is also valuable to distinguish between two often-confused words, *sterile* and *sterilize.* A *sterile* item is free of microbes (e.g., bacteria, molds, fungi, and spores). It has undergone *sterilization,* a process that renders the product sterile by destroying or removing viable microbes. The most common methods of sterilization include steam autoclaving, radiation, and terminal filtration.

TYPES OF STERILIZATION METHODS

Steam autoclaving, the "gold standard" of sterilization methods, is a process that utilizes pressurized steam (121°C for 20 min at 15 PSI) to destroy pathogens. This process is suitable for sterilizing certain pharmaceuticals, glassware, and other heat-stable items (*2,3–5*). The timed autoclave cycle is monitored by pressure and temperature sensors whose output is recorded on a strip chart recorder. A drying cycle is used to minimize risk of contamination. Biologic indicators, e.g., preparations of certain heat-resistant bacteria, are used to monitor the effectiveness of the sterilization process. Under the conditions described above, dry heat will not destroy spores and other heat-resistant pathogens; saturated steam is required to penetrate the spore walls and kill them. Dry heat is equally effective only at significantly higher temperatures and for prolonged heating cycles.

Sterilization by radiation involves placing the item to be sterilized in a very intense ^{60}Co gamma radiation field in order to deliver a dose of ~2.5 million rads. This procedure (*2,6–9*) is most suited for sterilization of syringes, needles, tubing, and other small disposable items. It is not routinely used to sterilize solutions. Bacterial spores are most resistant to radiation damage; however, the very high levels of radiation employed usually assure sterility in the presence of spores. Gram-negative rods are most sensitive to the effects of radiation. Other pathogens, e.g., yeasts and fungi, show sensitivities between these two extremes.

Sterilization by gaseous ethylene oxide (ETO) is an alternative for certain heat-sensitive items that cannot tolerate steam autoclaving. Ethylene oxide sterilization also is performed in a heated, pressurized chamber, but at a lower

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temperature, usually 30–70°C (*2,10,11,12*) and at a concentration of 10,000 mg/l. Under these conditions, ETO alkylates various reactive chemical groups in spore or vegetative cells, resulting in cell death. Sterilization by this technique also requires monitoring with biologic indicators to insure sterility. A wide variety of materials and equipment can be sterilized by this technique; the nutritional value of essential components in food, however, is partially or completely destroyed by ETO sterilization. Thus, animals fed ETO-sterilized food may fail to grow (*13,14*). Health concerns regarding items properly sterilized by the ETO method appear to be unfounded since the residual amount of ETO in sterilized items is quite small.

Terminal filtration in accordance with the guidelines set out in the USP XXI is performed by passing a solution to be sterilized through a 0.22-μm membrane filter (*2,15,16*). This is the method of choice for sterilization of heat-sensitive pharmaceuticals, e.g., radiopharmaceutical cold kits, antibiotics, and other drugs during the final stages of packaging or prior to lyophilization. In addition to quantitative filtration of bacteria and other pathogens, this procedure removes a wide variety of undesirable particulate matter often found in bulk preparations (*2*), such as colloids and particles of glass, rubber, carbon, cotton, lint, dust, and metal. Terminal filtration of solutions is relatively inexpensive, simple, rapid, and reliable and involves essentially no loss of product. It is also perfectly suited for sterilization of air, nitrogen, and other medical gases.

TESTING FOR STERILITY AND APYROGENICITY

According to the USP XXI, sterility tests are performed by the inoculation of growth media, storage of the inoculated media under conditions suitable for the growth of viable microorganisms, then checking the media for evidence of growth. Since sterilization is designed to eliminate pathogenic organisms completely from parenterally administered drugs, unlike the pyrogen test, it has no permissible level of pathogens. The only acceptable test result is "No Growth."

The pyrogen test is designed to limit to an acceptable level the risk of fever induction following injection of a pharmaceutical. Although one can readily produce pyrogen-free equipment and drug storage containers, it is virtually impossible to completely eliminate pyrogens from pharmaceutical preparations since chemicals also contain traces of pyrogens. Fortunately, trace levels from these sources (typically < 0.2 endotoxin units/ml of injectate) are too low to cause a fever response in patients (*1*).

For manufacturers of commercially available pharmaceuticals, radiopharmaceuticals, and cold kits, every lot of every pharmaceutical, radioactive or not, that is to be administered parenterally to a patient must undergo both sterility and pyrogen testing to insure patient safety. Nonradioactive pharmaceuticals manufactured by a hospital pharmacy (e.g., antibiotic solutions or other preparations designed for parenteral administration) must also, on a routine basis, undergo sterility and pyrogen testing to insure patient safety. For radiopharmaceuticals manufactured locally in a nuclear medicine department, e.g., ^{131}I-MIBG, ^{123}I-HIPDM, or radiolabeled monoclonal antibodies, sterility and pyrogen testing should be performed in accordance with USP XXI guidelines.

Due to the short physical half-lives of certain investigational radiopharmaceuticals and to the necessity of injecting material that is freshly prepared, the USP XXI permits *post facto* testing of these products. That is, one may inject the drug and then initiate these tests as soon as practicable after the injection. This is especially pertinent since, depending upon the type of materials and volumes used, sterility testing may take several days or several weeks to perform. On the other hand, pyrogen testing can be performed in < 2 hr (*17*) so injection of material would not necessarily be delayed.

METHODS OF STERILITY TESTING

The USP XXI method of performing sterility testing of pharmaceuticals requires inoculation of the product in both fluid thioglycollate and soybean-casein digest media (solutions or suspensions), both readily available commercially. Fluid thioglycollate provides ideal conditions for growth of aerobic and anaerobic bacteria (those that tend to proliferate in the presence of and the absence of oxygen, respectively). Soybean-casein digest medium supports growth of fungi and molds. The official sterility test requires observation of the cultures for 14 days, but most institutions use abbreviated versions of this schedule. The USP XXI requires that no growth be observed at any time point prior to termination of the test according to each institution's protocol. Under certain conditions outlined in the USP XXI, provision is made for retesting of a lot of material if a positive sterility test is obtained. This usually requires complete retesting of a larger number of vials than originally tested.

Another routinely performed sterility test uses the BACTEC System (*18*). A normally sterile body fluid, e.g., blood, is introduced into aerobic and/or anaerobic vials containing a ^{14}C substrate, usually glucose. The vials are incubated at 37°C for up to 6 days and, on a daily basis, the head-space in each vial is monitored for microbial metabolism by assaying the $^{14}CO_2$ levels. The amount of $^{14}CO_2$ is keyed to a growth index, which must exceed an established threshold level for a positive reading. Every vial resulting in a positive reading is then subcultured in order to isolate and identify the microbe present. False-negative studies have been identified with certain anaerobic species; false-positives with high levels of leukocytes in specimens.

METHODS OF PYROGEN TESTING

Pyrogen testing, as described in the USP XXI, requires that three healthy rabbits be injected intravenously with a specific volume of the drug to be tested, followed by monitoring of rectal temperatures for three hours afterwards. A positive test is recorded if an individual rabbit shows a temperature increase of ≥ 0.6°C above baseline temperature, or if the sum of the temperature elevations of all three rabbits exceeds

1.4°C. While some institutions perform the Rabbit Test at their own facility, more frequently it is performed by companies specializing in this type of testing.

The USP XXI also describes the Limulus Amoebacyte Lysate Test (LAL) for the presence of endotoxin. It is based on the F. B. Bang discovery in 1956 of extremely large numbers of dead horseshoe crabs on the South Atlantic Coast. After a thorough investigation, he ultimately published a paper (*19*) in which he recorded the observation that gram-negative infection of the horseshoe crab *limulus polyphemus* resulted in complete coagulation of the animal's circulatory system, causing death of the animal. Bang and Levin (*20*) later proved that this gelation of the blood was caused by reaction of bacterial endotoxin with a clottable protein in the circulating amoebocytes of limulus blood and ultimately developed a lysate of washed amoebocytes that proved to be extremely sensitive as an indicator of the presence of endotoxin. The turbimetric properties of this lysate have been incorporated into the currently available pyrogen test kits. The reaction has been shown by other investigators (*21,22, 23*) to be enzymatically mediated. Gram negative endotoxins are recognized as the most important source of pyrogen contamination.

The LAL test has been proven (*24*) to be at least five times as sensitive as the Rabbit Test for detecting low levels of pyrogens and has proven very useful in determining if small equipment (tubing, probes, etc) is free of surface contamination. This increased sensitivity makes the LAL Test particularly desirable for testing drugs to be administered intrathecally because endotoxin introduced directly into cerebral spinal fluid is at least 1000 times as potent as that administered intravenously (*24*).

In 1975, Cooper and co-workers (*25*) reported on the ability of the LAL test to identify endotoxin in several commercial preparations of ^{131}I HSA (RISA), which had caused adverse reactions, including aseptic meningitis, in patients receiving intrathecal injections of this radiopharmaceutical. The Rabbit Test for pyrogens was negative on a dose/weight basis. The source of endotoxin was ultimately traced to a phosphate buffer component used in the manufacturing process.

The pyrogen test requires simultaneous incubation in pyrogen-free glass tubes of a negative control (pyrogen-free saline), a positive control (endotoxin solution), the sample, and the sample spiked with endotoxin. The required conditions are incubation for up to 60 min in a water bath maintained at 37°C with no sample agitation. The spiked sample is necessary to insure that it is possible to obtain a positive test with a particular sample. Some compounds, e.g., blood, solutions with high osmolality (salt concentration), or those containing heavy metals must be diluted significantly prior to testing, or a positive result cannot be obtained, even when endotoxin is present (*16*). These samples probably interfere by poisoning the operative enzyme system. In addition, it is generally necessary to adjust the pH of the sample to within a range of 6.0–8.0 to insure that a positive test can be obtained. This must be done with pyrogen-free sodium hydroxide or pyrogen-free hydrochloric acid solution.

Table 1 outlines sample preparation for performing the pyrogen test. All sample preparation is performed in a laminar flow hood using aseptic technique. The LAL Test is positive if contents of the tubes containing the positive control, the sample, and the sample spiked with endotoxin all form a firm gel following incubation with LAL reagent in a water bath at 37°C for 60 ± 1 min.

TECHNIQUES FOR PYROGEN REMOVAL

In order to produce pyrogen-free pharmaceuticals, it is mandatory to use only sterile, pyrogen-free reagents, solvents, disposables and equipment. In addition, only containers known to be pyrogen-free may be used for drug storage. Equipment and disposables should be handled only with sterile gloves and all work should be performed in a laminar flow hood equipped with a HEPA (High Efficiency Particulate Attenuation) filter. This filter traps all particulates ≥ 3 μm in diameter. Since pathogens are typically larger than this limit, sterile, particulate-free air is produced. Direction of flow of this sterile air prevents product contamination by room air.

Often it is necessary to depyrogenate glassware and other heat-stable equipment. This is accomplished by dry heat for 2 hr at 190°C. Glass vials and bottles of all sizes are routinely depyrogenated by pharmaceutical manufacturers prior to filling them with their final product in order to eliminate a potential source of pyrogenic reactions. For solutions with low-level pyrogen contamination, affinity chromatography techniques are available (*26,27*), which effectively remove significant quantities of pyrogens. In addition, commercially available filters* are capable of removing pyrogens by an adsorption process (*28,29*). While these procedures do not completely eliminate soluble pyrogens, the level of pyrogens may be reduced sufficiently to pass the LAL or USP Rabbit Test for pyrogens.

CONCLUSION

Pharmaceutical manufacturers are required to perform sterility and pyrogen tests on a routine basis prior to shipping any pharmaceutical products, thereby relieving the consumer of the responsibility of sterility and pyrogen testing. The exception to this rule is the case where an investigational radiopharmaceutical is formulated from chemicals and/or radiochemicals in a nuclear medicine department, in which case the preparer is responsible for testing for sterility, apyrogenicity, physical appearance, pH, radiochemical purity, fill

TABLE 1. Sample Preparation for Performing the Pyrogen Test

Tube #	Sample	Volume of LAL	Volume/Sample
1-2	Negative control	0.1 ml	0.1 ml 0.9% saline (inj)
3-4	Positive control	0.1 ml	0.1 ml endotoxin solution
5-6	Test sample	0.1 ml	0.1 ml test sample
7-8	Test sample + endotoxin	0.1 ml	0.1 ml test sample + 0.1 ml endotoxin solution

volume, and perhaps other parameters. For NDA-approved parenteral drugs, the final product quality depends upon the cooperation of drug manufacturers in maintaining aseptic conditions during the manufacturing process as well as enforcement of Good Manufacturing Practices by the FDA.

Healthcare workers should recognize the efforts of manufacturers of all parenterally administered pharmaceuticals and the superior job they have done in complying with current regulations related to sterility and pyrogen testing. The evidence for drug safety in this country with regard not only to pyrogen and sterility testing, but also to other safety factors, has been unparalleled and will continue to be so due to the high sensitivity of these test procedures and to the diligence of the manufacturers in performing them.

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NOTE

* Posidyne Membrane, Pall Corp., East Hill, NY.

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