

The United States Pharmacopeia and Depyrogenation

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Introduction

For many years chapters in the United States Pharmacopeia (USP) have made reference to depyrogenation. These chapters include <85> Bacterial Endotoxins Test (BET),¹ <797> Pharmaceutical Compounding of Sterile Preparations and the general information chapter,² <1211> Sterility and Sterility Assurance of Compendial Articles.³ More recently the USP introduced a monograph on Endotoxin Indicator for Depyrogenation.⁴ Currently the USP is in the process of introducing a new set of general information chapters dedicated to the subject. These comprise a main (parent) chapter <1228> Depyrogenation,⁵ and (currently) three sub-chapters: <1228.1> Dry Heat Depyrogenation ; <1228.3> Depyrogenation by Filtration⁷ and <1228.5> Endotoxin Indicators for Depyrogenation,⁶ the last of which became official in the first supplement to USP 40 on May 1, 2017.

The objective of this article is to frame in one document all of the various USP chapters in order to provide context around depyrogenation, how the pharmacopeia has evolved on this subject and illuminate the similarities and differences among the different chapters. Readers are cautioned to always read the original documents to assure regulatory compliance for their own unique situations.

General Test and Assays

Chapters in the General Test and Assays, which have numbers below <1000>, set standards that are to be adhered to when referenced unless alternatives have been validated according to the provisions of the General Notices section of the USP.

USP <85> Bacterial Endotoxin Testing¹

This chapter includes the requirement to depyrogenate all glassware and other heat-stable materials in a hot air oven using a validated process. It notes that a commonly used minimum time and temperature is 30 min at 250°C.

USP <797> Pharmaceutical Compounding of Sterile Preparations²

This chapter states "Dry heat depyrogenation shall be used to render glassware or containers such as vials free from pyrogens as well as viable microbes. A typical cycle would be 30 minutes at 250°. The description of the dry heat depyrogenation cycle and duration for specific load items shall be included in written documentation in the compounding facility. The effectiveness of the dry heat depyrogenation cycle shall be verified using endotoxin challenge vials (ECVs). The bacterial endotoxin test should be performed on the ECVs to verify that the cycle is capable of achieving a 3-log reduction in endotoxin (see Sterilization and Sterility Assurance of Compendial Articles <1211>³ and Bacterial Endotoxins Test <85>¹)."

General Information

USP general information chapters, which do not establish standards, are numbered between <1000> and <1999>. They provide advice and guidance on good practices. Informational chapters that address depyrogenation include:

USP <1211> Sterility and Sterility Assurance of Compendial Articles³

This chapter includes a section titled Dry-Heat Sterilization/Depyrogenation. It describes dry heat ovens (for batch processing) and tunnel ovens and notes that they utilize heated, HEPA filtered air, distributed by convection or radiation and a blower system and a system for controlling critical parameters. It notes that the temperature may vary by $\pm 15^\circ$ in an empty chamber at 250°C or above.

The section states that because of a much shorter dwell time in a tunnel system, the temperature is usually much higher than a batch process oven. This is usually followed by rapid cooling prior to the aseptic filling operation. The section stresses the importance of assuring exposure to the process temperature for sufficient time. It notes that since dry heat depyrogenation is a more rigorous process than sterilization, it is generally not necessary to include biological indicators (for sterility assurance) if depyrogenation is validated. The section also states that an endotoxin reduction of at least 3 log is a suitable acceptance criterion for depyrogenation that will also ensure sterilization. It mentions testing articles inoculated with reference standard endotoxin (RSE) for residual endotoxin after they have been processed and refers to the BET chapter, <85>.¹

Notably, there is a new series of USP informational (non-binding) chapters on depyrogenation, which include:

USP <1228> Depyrogenation⁵

The chapter provides an overview of depyrogenation. The introductory section defines depyrogenation and refers to sub-chapters in the <1228> series (currently <1228.1>,⁶ <1228.3>⁷ and <1228.5>.⁸). The introduction cross references the USP chapter <1211> Sterilization and Sterility Assurance of Compendial Articles³ which was a principle (but limited) source of information on the subject until the publication of chapter <1228> Depyrogenation.⁵ It emphasizes the importance of risk management tools in controlling endotoxin contamination and states that the <1228> series of chapters may be considered alternatives to traditional approaches.

The next section discusses the nature of endotoxin and distinguishes between the native endotoxin that is likely to contaminate product and the purified lipopolysaccharides that are used as standards in testing.

This is followed by a section titled Measuring Endotoxin Pre- and Post-Processing, which refers to testing of endotoxin indicators (EIs) before and after they have been subject to a depyrogenation process. It refers to the BET chapter <85>¹ for testing. Preparation of EIs is discussed with considerations of appropriate carrier type, source of the challenge endotoxin and the dose (level of activity) to be used. For recovery of endotoxin from challenge articles for testing, this section refers to USP chapter <161> Medical Devices—Bacterial Endotoxin and Pyrogen Tests and to the standard ANSI/AAMI ST72:2011.¹⁰ It also allows for development and validation of a recovery method that better suits the material under test.

The measurement section includes a discussion of the degree of endotoxin reduction in a depyrogenation process. It notes that historically process efficiency has been assessed in terms of logarithmic (log) reduction of the endotoxin added to the carrier prior to treatment. Typically at least a three log-reduction has been expected. Chapter <1228>⁵ suggests that greater or lesser levels of reduction may be appropriate depending on the materials and the process; it notes that the chosen level of reduction should be justified.

A section on Control of Endotoxin in Parenteral Products notes that the best way to minimize endotoxin contamination is to control the Gram-negative bacterial bioburden in raw materials, equipment, process streams, and the manufacturing environment, including operators. It refers to three categories of control, which are: indirect control, (minimization of endotoxin inputs to the process), process control (validated/qualified controls to minimize endotoxin input during the manufacturing process) and direct control (validated destruction or removal of endotoxins – i.e. depyrogenation, which is the subject of the <1228> series of chapters). Each of these three categories is then discussed in turn.

This is followed by sections on Selection of an Appropriate Depyrogenation Method, Validation of a Depyrogenation Method, Routine Process Control (which includes verification of ongoing process control and periodic confirmation of the effectiveness of depyrogenation processes) and Routine Testing. These lay out general principles appropriate to their subject.

<1228.1> Dry Heat Depyrogenation⁶

The introductory section to the chapter begins by stating that dry heat is the method most frequently used to depyrogenate heat stable materials and that it is dependent upon two parameters: time and temperature. The kinetics of endotoxin destruction by dry heat are briefly discussed (and are addressed in more detail later in the section on Depyrogenation Process Control). Consistent with USP <1211>³, the introduction notes that the temperatures used for dry heat depyrogenation are generally higher than those used for dry heat sterilization because endotoxins are more resistant to heat than bacterial spores.

The introduction is followed by a section on Technologies Used for Depyrogenation by Dry Heat, which notes that there are two major categories of equipment: dry heat “batch” ovens and continuous tunnel systems. Tunnel ovens are widely used for processing vials or bottles as part of a flow through system following a washer. After a cooling step the vials typically exit from the tunnel system on a conveyor into a filling suite where they are filled with product. Depyrogenation ovens require a control of several operational parameters, principally time and temperature. Ovens commonly incorporate air circulation systems to minimize temperature gradients and HEPA filters for recirculated and incoming air to control particulates. Most depyrogenation ovens for batch processing are convection ovens; tunnel ovens commonly utilize radiant heat as well as convection. Dedicated sub-sections on batch ovens and on continuous tunnels provide additional information on each.

A paragraph titled Dry Heat Depyrogenation Fundamentals explains that the limited heat capacity of dry air results in relatively slow heating and cooling of the load and notes the variability in temperature distribution in dry heat ovens and tunnels. Consequently, oven loading pattern is important and items in ovens should be placed in the same locations as confirmed acceptable in validation. Changing the load configuration can alter the air flow and the exposure of the control probe and result in process variability.

The section on Depyrogenation Process Control describes the calculation of an F_D value, which is a function measuring the depyrogenation effect under defined conditions. In this case an F_D of 1 is an effect equivalent to that obtained by exposure to a temperature of 250°C for 1 minute. F_D is to depyrogenation as what F_0 is to steam sterilization.

The section on Validation notes that the temperatures and time exposure of dry heat depyrogenation can challenge material integrity and stability. It recommends determination of both the endotoxin load on incoming

materials and the endotoxin reduction required to assure patient safety before validation. A detailed description of validation steps follows in subsections under the following headings:

- Equipment Qualification (EQ): EQ may be separated into installation qualification (IQ) and operational qualification (OQ)
- Empty Chamber Temperature Distribution for Ovens
- Empty Temperature Distribution in Tunnels: The chapter states that such studies are of limited value because an empty tunnel always produces much more variability than a loaded tunnel.
- Component Mapping: This comprises measurement of heating of load items under load and operational conditions.
- Load Mapping in Ovens
- Load Mapping in Tunnels
- Confirmation of Depyrogenation: Perhaps surprisingly, the chapter suggests that an endotoxin challenge may not be necessary in some cases. Recommendations are made for the minimum number of samples to be taken for endotoxin testing. It states that the process is considered acceptable if the amount of residual endotoxin measured per sample is NMT 0.1 EU.

A final section of the main text addresses Routine Process Control, which stresses the importance of ongoing controls to assure that the system continues to operate in the validated state. It refers back to the section of the same name in the parent chapter, <1228>⁵.

USP <1228.3> Depyrogenation by Filtration⁷

The introduction defines filtration and discusses particular considerations for solutions containing proteins and peptides. The different types of filter media are briefly reviewed. These are then discussed in more detail in the subsequent section on Technologies Used for Depyrogenation by Filtration, which comprises the following subsections, each describing a technology and its application for depyrogenation:

- Microporous Membrane Filtration: Although the nominal pore size is too large to retain endotoxins, such filters may have surface properties (positive charge, hydrophobic) that remove endotoxins by adsorption.
- Reverse Osmosis: Reverse osmosis membranes do exclude endotoxin. However, they can be contaminated by bacteria and thus contribute endotoxin to the system.
- Ultrafiltration: After a description of ultrafilters, it is noted that they can be effective in depyrogenation of solutions containing small molecules, they are not suitable for proteins that are retained by the membrane together with the endotoxin. Some ultrafilters also remove endotoxins by adsorption.
- Charge-Modified Depth Filters: As with microporous membranes, the nominal pore size is too large to retain endotoxins but charge modified membranes can be effective in removing endotoxins by adsorption. It is noted that cellulosic depth filters can contribute β -1,3-glucans to the solution being processed, which can activate the LAL reagent used in a BET to give a false positive or enhanced result.

- Membrane Adsorbers: The principles and drawbacks of ion exchange chromatography are reviewed. Depyrogenation of proteins can be accomplished by either removing endotoxin from the protein or by removing the protein from the endotoxin, depending on the nature of the protein. Removal of endotoxin by anionic and hydrophobic mixed-mode membrane adsorbers is also mentioned.

The final section on validation simply refers to the main chapter, Depyrogenation <1228>⁵.

USP <1228.5> Endotoxin Indicators for Depyrogenation⁸

The introductory section defines depyrogenation (with reference to the parent chapter, <1228> Depyrogenation⁵), endotoxin and endotoxin indicators (EIs). In addition to qualification of dry heat depyrogenation, Els can be used for qualifying endotoxin removal by washing, rinsing, cleaning, and filtration technologies. It notes that carriers can be a range of materials, from rubber stoppers to bulk product and stainless steel coupons. Finally, the section introduces laboratory-derived endotoxin for Els prepared in-house.

The next section draws a distinction between endotoxin and LPS and notes that the properties of purified LPS may differ from those of the unpurified endotoxins that can contaminate products. It states that excipients (fillers) that may be in control standard endotoxin (CSE) preparations can reduce the heat resistance of LPS and may affect its recovery [from a carrier].

A section on application of endotoxin indicators states that Els, including the carrier being used as a substrate for the endotoxin, should be appropriate for the process that is being validated. In the case of a material (e.g. raw material or in-process material) that is significantly contaminated with endotoxin, it may not be necessary to add endotoxin in order to show sufficient endotoxin removal to qualify the process. (Although not explicitly stated in <1228.5>, in such cases an EI is not required.) For materials that are not contaminated, the sections suggest that use of purified LPS preparations may not reflect the potential of the depyrogenation process to remove the unpurified endotoxins that are the potential contaminant. It proposes that endotoxin preparations made from Gram-negative cultures may present more realistic challenge to the process. The section concludes by recommending that endotoxin not be introduced into an actual manufacturing process in order to demonstrate removal; the use of a pilot or laboratory scale process is suggested.

The section on preparation and use of endotoxin indicators presents some considerations for preparing an in-house laboratory prepared endotoxin (to be used to create Els), noting that there is no standard method for doing so. (Note that although it is not referenced in <1228.5>, the USP monograph, Endotoxin Indicator for Depyrogenation, specifies the use of purified LPS for Els.) The preparation and use section suggests using as a source of laboratory-prepared endotoxins either a bacterial strain from a recognized culture collection or a well characterized Gram-negative isolate from the facility, water system, or process material or product. The procedures for preparation of all aspects of preparation of Els (including culture maintenance) should be appropriately documented in procedures and records. The activity of in-house endotoxin preparations should be determined relative to an endotoxin standard that is traceable to RSE. This section also recommends that the slope and y-intercept for a series of dilutions of in-house endotoxin preparations in [photometric] endotoxin tests be compared for consistency with those of RSE or CSE. Finally, stability studies should be conducted; storage conditions for endotoxin preparations should be conducted and expiration dates should be established.

The section on inoculation of EIs describes how endotoxin is added to carriers, which is commonly by pipetting a small volume of highly concentrated endotoxin on the carrier. In the case of dry carriers, such as glass vials or coupons, the endotoxin is fixed by drying it. For liquid carriers the section recommends that the level of inoculation be a worst case high concentration and that the selected concentration be justified.

The discussion of recovery of endotoxin from EIs notes that recoveries are often not 100% of that expected based on the inoculation. (It is noted that for commercially available EIs there should be little difficulty in achieving recovery within a factor of 2 of the labeled concentration.) This section recommends determining the percentage recovery of endotoxin added to in-house EI (dry or liquid) relative to the amount of endotoxin added in the inoculum. For recovery from in-house dry EIs the USP medical devices chapter, <161>⁹, ANSI/AAMI standard ST72:2011¹⁰ and a publication¹¹ are referenced. For liquids, this section recommends determining the stability of the added endotoxin over an appropriate period. The method adopted for endotoxin recovery should be verified for consistency and then used in subsequent studies to assure comparability of results.

A section on the test methodology for the analysis of EIs refers to the USP BET chapter, <85>¹. This is followed by a section addressing the analysis of results of depyrogenation studies. This describes how the effectiveness of a depyrogenation process is assessed by comparing the endotoxin activity of processed EIs to that of control EIs that have not been subject to depyrogenation. This is commonly presented in terms of the log reduction [log base 10 assumed] and an example is given. The section observes that historically a reduction of at least 3 logs has been expected and states that this may be excessive in some cases but may not be sufficient in other. (However, compounding pharmacists should note that USP chapter <797> Pharmaceutical Compounding of Sterile Preparations², which is not an advisory chapter, specifies that “the [depyrogenation] cycle is capable of achieving a 3-log reduction in endotoxin.” This caution is not given in <1228.5>.) The test methodology section also suggests that sometimes it may not be necessary to spike with 1000 EU to demonstrate a 3-log reduction. This contrasts with the USP monograph, Endotoxin Indicator for Depyrogenation⁴, which is discussed below and is a standard for EIs (i.e. it is mandatory if referenced); the monograph specifies that “the amount of endotoxin on the carrier is sufficient to allow recovery at least 1000 EU per carrier.”

Monographs

Monographs establish standards for specific articles. There is only one USP monograph that addresses depyrogenation, which is discussed below. (Note that the title is singular, contrasting with the similar, but plural, title of <1228.5>⁸.)

*Endotoxin Indicator for Depyrogenation*⁴

The definition section of the monograph describes endotoxin indicators. It specifies that the endotoxin be a purified lipopolysaccharide validated for use in or on an EI. The carrier must be “... a material appropriate for the intended depyrogenation processes...” to which it will be subjected. The amount of endotoxin on the carrier is sufficient to allow recovery at least 1000 EU per carrier.

The subsequent identification section requires that the endotoxin has equivalent characteristics to USP Endotoxin RS. A section on performance tests includes subsections on the carrier and endotoxin recovery tests. The carrier must be depyrogenated or of known

endotoxin level before adding the endotoxin challenge. It should be the same as or chemically similar to the surface or material used for measuring depyrogenation (which presumably means similar to the materials to be subject to the depyrogenation process). The subsection on endotoxin recovery tests refers to the BET chapter 1 and requires that the endotoxin concentration determined for the [unprocessed] EI be within a factor of 2 of the labeled concentration.

A section on specific tests requires tests for purity that specify absence of substances enhance the test, including glucans, and the absence of filler for the endotoxin, which might result in enhanced or inhibited depyrogenation effects.

Finally, an additional requirements section addresses packaging and storage, expiration dating, labeling disposal and USP reference standards.

Summary

The USP includes a number of references to depyrogenation. More recently, starting with the monograph, Endotoxin Indicator for Depyrogenation, chapters have been added that are dedicated to the subject. With the new <1228> series of chapters there is now a lot of information depyrogenation in the pharmacopeia. This article summarizes these chapters and distinguishes between standards and the informational chapters. This distinction is important. USP chapters below <1000> and monographs set standards which must be followed if they are referenced (unless an alternative has been appropriately validated). The general information chapters, with numbers between <1000> and <1999> (which includes the <1228> depyrogenation series) provide general information and are advisory; they are not mandatory.

References

- <85> Bacterial Endotoxins Test. 2016. USP 40-NF 35. 163. United States Pharmacopeial Convention, Rockville, MD.
- <797> Pharmaceutical Compounding of Sterile Preparations 2016. USP 40-NF 35. 683. United States Pharmacopeial Convention, Rockville, MD.
- <1211> Sterility and Sterility Assurance of Compendial Articles. 2016. USP 40-NF 35. 1744. United States Pharmacopeial Convention, Rockville, MD.
- Endotoxin Indicator for Depyrogenation. 2016. USP 40-NF 35. 3977. United States Pharmacopeial Convention, Rockville, MD.
- <1228> Depyrogenation. 2016. USP 40-NF 35. United States Pharmacopeial Convention, Rockville, MD.
- <1228.1> Dry Heat Depyrogenation. USP 40-NF 35. United States Pharmacopeial Convention, Rockville, MD.
- <1228.3> Depyrogenation by Filtration. USP 40-NF 35. United States Pharmacopeial Convention, Rockville, MD.
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- ANSI/AAMI ST72:2011, Bacterial endotoxins - Test methodologies, routine monitoring and alternatives to batch testing. Association for the Advancement of Medical Instrumentation, Arlington, VA.
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