A Practical Approach to Depyrogenation Studies Using Bacterial Endotoxin

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Depyrogenation devices, such as tunnels, are used in the pharmaceutical industry to prepare components for aseptic filling. To qualify such devices, various pharmacopeias require depyrogenation devices to be periodically challenged with high levels of bacterial endotoxin. Although the pharmacopeias state the acceptance criteria, little consideration is given to the practical approach. This article discusses the theoretical concept of depyrogenation. A case study of a depyrogenation tunnel is used to define some of the practical aspects of a depyrogenation study that need to be considered.

INTRODUCTION

The bacterial endotoxin test (BET) is a relatively straightforward test and has been a pharmacopeial method since 1980, when it first appeared in the *United States Pharmacopeia* (USP). The test, using *Limulus* amebocyte lysate (LAL) methodology, is described in detail in the harmonized chapters in both the European Pharmacopoeia (1) and the United States Pharmacopeia (2). The test describes the detection of the most common and significant pyrogenic material found in pharmaceutical production: gram-negative bacterial endotoxin. LAL is an extract from the lysed blood cells (amebocytes) of the horseshoe crab *Limulus polyphemus*, or related species.

The need to perform the LAL test for endotoxins is well established. Endotoxins can cause, to varying degrees depending upon potency and target site, endotoxemia (i.e., the presence of bacterial toxins in the blood) and septic shock (i.e., the prolonged presence of bacteria and bacterial toxins in the body). The effects of endotoxin in the human body include high fever, vasodilatation, diarrhea, and fetal shock syndrome. Due to the level of risk, pharmaceutical water systems and parenteral products are tested for pyrogens including, or exclusively, endotoxins (3).

Despite the relative comprehensiveness of the pharmacopeial monographs for the LAL test, one key application of endotoxin testing is not described in great detail: conducting depyrogenation studies. A depyrogenation study is the key biological test, in addition to thermometric tests, for the qualification of depyrogenation devices. Depyrogenation can be defined as the elimination of all pyrogenic substances, including bacterial endotoxin, and is generally achieved by removal or inactivation (4). Depyrogenation, like sterilization, is an absolute term that can only be theoretically demonstrated because of test insensitivity.

Some scientists regard depyrogenation purely as endotoxin destruction or inactivation, and endotoxin removal as a distinct and unrelated process. Here the former refers to inactivating or destroying any endotoxin present on a component, the latter to the removal of any endotoxin present (5). With depyrogenation inactivation, the total destruction of the "pyroburden" is assumed; with endotoxin removal it is assumed that a significant portion of the pyroburden has been removed. Other scientists consider both processes to be part of depyrogenation.

This article examines the mechanism of endotoxin inactivation by dry heat and the practical steps to be taken for conducting a depyrogenation study. Depyrogenation of glassware is important in the production of parenteral pharmaceuticals as residual pyrogens could ultimately be injected into a patient resulting in an adverse reaction. This is especially important as endotox-

ins are heat stable, making them resistant to most conventional sterilization processes and thus necessitating separate tests for viable cells and endotoxin (6).

The assessment of depyrogenation involves the introduction of purified endotoxin of a high potency and post-process testing to assess if a minimum of a three-log reduction has been achieved. The concept is similar to that of steam sterilization studies, where a much higher level of microorganisms than would ever be found in the normal environment is introduced into a device as a worst-case challenge. In the case of steam sterilization, this is *Geobacillus stearothermophilus*. Depyrogenation follows a pattern similar to steam sterilization, in that the log-reduction will follow a linear plot (i.e., as time and temperature increase, the endotoxin level decreases). After this destruction continues to occur, it does not necessarily follow linear regression (i.e., it does not produce the same semi-log reduction seen for bioburden reduction in sterilization devices and becomes more noticeable at lower temperatures) and instead follows a biphasic reduction (7).

DEPYROGENATION

Before focusing on the practical aspects of depyrogenation studies, it is appropriate to consider the process of depyrogenation. The following are various mechanisms by which depyrogenation is achieved (8-11):

- Ultrafiltration—the process works by excluding endotoxin by molecular weight using an ultra-fine filter that blocks molecules of 10,000 Daltons or greater. This is often coupled with 0.1µm filter.
- Reverse osmosis—primarily functions as a size-excluding filter operating under a highly-pressurized condition. It will block 99.5% of endotoxin and ions or salts, but allow water molecules through. USP reverse osmosis (RO) can be used to make water for injection (WFI) (whereas to meet the European Pharmacopoeia requirement it can only be produced by distillation); within Europe it is used to produce highly-purified water.
- Affinity chromatography (e.g., Diethylaminoethyl cellulose [DEAE] sepharose or polymyxin-B, which binds endotoxin by using a positive charge to attract the negatively-charged endotoxin and then allowing its elution)—such processes are affected by the pH range, temperature, flow rate, and amount of electrolytes in the solution.
- Dilution or rinsing—the amount of endotoxin is washed away or reduced using WFI.
- Distillation—functions by turning water from a liquid to a vapor and then from vapor back to liquid. Endotoxin is removed by the rapid boiling that causes the water molecules to evaporate and the relatively larger lipopolysaccharide (LPS) molecules to remain behind.
- Adsorption (e.g., activated carbon beds, where endotoxin is adsorbed into charcoal, or depth filters)—this functions by attracting negatively-charged endotoxin molecules to the carbon bed. This mechanism is only efficient to a small degree and is affected by a range of environmental factors.
- Hydrophobic attachment—certain materials, like polyethylene, can bind endotoxin.
- Acid or base hydrolysis—this destroys the eight-carbon sugar: two-keto-three-deoxyoctonic acid that links Lipid-A to the core polysaccharide and causes physiochemical changes that decrease pyrogenicity. The separated Lipid-A loses its pyrogenic activity). An example is 0.05M HCl for 30 min. at 100 °C or 0.5 M NaOH at 50 °C for 30 min.

  It is possible, with alkaline hydrolysis, that the level of endotoxin may initially rise as part of the separation process.

  The hydrolysis methods are frequently used for depyrogenating glassware. The efficiency of this process is often connected to the cleanliness of the glassware prior to treatment.
- Oxidation—works by peroxidation of the fatty acid in the Lipid-A region (e.g., using hydrogen peroxide).
- Ionizing radiation—a very slow and inconsistent process.
- Ethylene oxide—functions by nucleophilic substitution in the glucosomne of Lipid-A. It is not the most efficient depyrogenation process, and where endotoxin inactivation occurs this is normally a side effect of sterilization.
- Moist heat—conventional autoclaving will not destroy endotoxin. However, the combination of

a chemical additive (e.g., hydrogen peroxide) and physical variations (e.g., five hours at 121°C with a pressure of 20 PSI and a pH of 3.8) are sometimes effective. Alternatively, some studies have shown that autoclaving for prolonged periods of time or at higher temperatures can be effective at reducing endotoxin and other pyrogenic substances (12, 13).

- Endotoxin can be inactivated by wet heat, although this is only effective with far lower concentrations of endotoxin and is applied to non-heat stable materials. Destruction of endotoxin is far more difficult to achieve, and lower log reductions when compared with dry heat are achieved (14).
- Dry heat (physical destruction), such as convection (transfer of heat by movement of fluid or air), conduction (transfer of heat from adjacent molecules), or irradiation (emission of heat by electromagnetic radiation).

This paper primarily focuses on one method of inactivation: by dry heat. The qualification of a depyrogenation device operating by dry heat involves challenging the device, such as a depyrogenation tunnel, with a known level of purified endotoxin (LPS). This is sometimes described as an "endotoxin indicator," which is analogous to the "biological indicator" used to test a sterilization device like an autoclave (15).

Depyrogenation by Dry Heat—Case Study

The following is a case study of depyrogenation of glass vials in a hot air tunnel sterilizer.

Depyrogenation is an important part of the manufacture of pharmaceutical products and is distinct from sterilization. Sterilization refers to the destruction of living cells. However, the process does not necessarily destroy microbial by-products and toxins. Endotoxin is one toxin that is extremely heat stable and is not destroyed by standard sterilization cycles (e.g., autoclaving). If only sterilization is required to be demonstrated, this can be achieved using biological indicators impregnated with endospores from a heat resistant bacteria (e.g., *Bacillus subtilis var. niger* [often used for dry heat] or *Geobacillus stearothermophilus* [often used for moist heat, although the microorganism also has a high resistance to dry heat] [16]).

Various items can be depyrogenated by dry heat. The main example referred within this paper is glass vials used for the filling of parenteral product, notwithstanding that many of the principles might apply to other primary packaging materials providing that they can withstand high temperatures.

Depyrogenation by dry heat for glass in the pharmaceutical industry is the primary endotoxin destruction method used (17). This process both sterilizes and depyrogenates and is mainly used for glass components. Dry heat involves subjecting the components to a high level of heat (normally between 180 and 250°C) for a defined time (the higher the temperature, the shorter the time required). The typical cycle is 250°C for not less than 30 minutes. For example, the *European Pharmacopoeia* in chapter 2.6.8 states two possible time-temperature combinations for depyrogenation: 60 minutes at 200°C or 30 minutes at 250°C. A quantity of endotoxin destroyed at 250°C for 60 minutes would not necessarily be totally destroyed at 200°C at 60 minutes, based on the non-linearity of the thermal destruction curve. Endotoxin destruction at low temperature is of the second-order (18).

Depyrogenation dry heat devices include ovens and tunnel sterilizers. To operate, depyrogenation devices require a series of parameters to be controlled. These parameters include laminar airflow controlled by high-efficiency particulate air (HEPA) filters, with a specification for air velocity and particulates. Where the device is a depyrogenation tunnel, the rate of speed (e.g., minimum, maximum, and nominal) must be measured and verified. The key function for depyrogenation is temperature control. Such depyrogenation devices require qualifying as part of validation. This is performed along the familiar lines of design qualification, installation qualification, operational qualification, and performance qualification, as well as annual re-qualifications. A depyrogenation study is a test of the physical capabilities of a device to depyrogenate an article or device. It is demonstrated by physical measurements (including temperature) and biological (using bacterial endotoxin). The biological test is the concern of this paper.

As part of the validation, normally at the performance qualification stage, depyrogenation devices are biologi-

cally challenged using a known level of a high concentration of *Escherichia coli* endotoxin. The preparation used is a freeze-dried extract from the Gram-negative bacterial cell wall lipopolysaccharide (LPS). The preparation is similar to the control standard endotoxin (CSE) used for routine LAL testing, although the concentration, once reconstituted, is far greater.

As indicated earlier, the mechanism for endotoxin destruction by depyrogenation is not fully understood (19). Depyrogenation by dry heat follows what Ludwig and Avis (18) describe as a "biphasic destruction curve." This is a logarithmic reduction until three logs of endotoxin have been eliminated and is a varied slow down when the inactivation of endotoxin ceases to decrease at a log-step rate. This is partially the reason why the criteria for a successful depyrogenation validation are set at a minimum of three-logs to provide an over-kill and companies go further and stipulate a minimum of four-logs (20). The time and temperature combination is of importance. Achieving a three-log reduction at 170°C may take 100 min. under one set of conditions. Whereas by increasing the temperature to 250°C, the time would theoretically be reduced to approximately 30 min.

A further variability, outside of the theoretical approach of such studies, would be the different potencies and thermal death of environmental endotoxin from different Gram-negative bacterial lipopolysaccharide (21).

Although the first ever depyrogenation studies were conducted in the early 1940s using rabbits to detect residual pyrogens, it is not considered ethical, or scientifically valid, to use the rabbit test for depyrogenation studies. Any of the three established LAL methods can be used to conduct a depyrogenation study: gel-clot, turbidimetric, and chromogenic. However, where possible, the gel-clot method is not recommended because of the large number of dilutions required.

The endotoxin used in the study should be licensed by the US Food and Drug Administration and purchased with a certificate of analysis for the lot of lysate used, with the potency determination having been undertaken by the supplier against reference standard endotoxin. This potency is used to convert the labeled weight of the endotoxin (expressed in nanograms) into Endotoxin Units (expressed as *X* EU/ng).

The testing of a depyrogenation device, at performance qualification, firstly involves running the device with a full set of containers in normal operation. Simultaneously, the depyrogenation device is temperature mapped using thermocouples. The thermocouples indicate where the cold spots (i.e., areas of the lowest temperatures) are within the device. The run is then repeated using endotoxin challenges at these colder areas, alongside thermocouples. One problem to consider, before undertaking such a study, is how to identify the inoculated vials once they enter the tunnel. Many tunnel sterilizers have a capacity for many thousands of bottles. A second consideration is the number of challenge vials to use in the study. The number used will be based on the size of the depyrogenation device. Typically, five to ten challenge vials are sufficient to assess the depyrogenation capabilities of the device being tested.

The following are two approaches for adding the endotoxin challenges:

- Using a high potency endotoxin spike directly onto the surface of the container to be depyrogenated; allowing this to dry or to freeze dry and then placing it into the depyrogenation device
- Using vials of high concentration endotoxin and substituting these for the containers.

Of the two methods, the former is the one that is closer to a representative challenge. The second approach, due to the endotoxin being contained in a vial of a different size (and invariably of a different type of glass), is less of a representative challenge. The second method is not often accepted by regulatory authorities.

In both cases the endotoxin challenge is typically 1000 Endotoxin Units (EU) or greater. The level of the challenge is determined by using control vials that are not subjected to the depyrogenation cycle. These are tested alongside the test vials on completion of the depyrogenation run. It is not necessary to recover all of the endotoxin from the control vials, and it is acknowledged that recovering endotoxin is difficult. A study by Plant gave a range of between 20 and 70% as the typical recovery range (22). It is only necessary to recover a level of endotoxin to show that the necessary log reduction has been achieved. Therefore, if a three-log reduction was required and 1000 EU were the theoretical chal-

lenge per container, but only 500 EU per container were recovered, then provided the test vials showed <0.5 EU per device recovery, the three-log reduction would have been achieved. Nevertheless, some level of laboratory competence should be demonstrated, in that the level of endotoxin recovered from control vials can be done so reproducibly.

When carrying out a depyrogenation study, pyrogen-free pipettes and glassware must be used throughout. Exposed vials should also be covered with a pyrogen-free covering, such as Parafilm-M (Pechiney Plastic Packaging). The vials that are challenged with endotoxin require preparing the day before the validation run in order to allow for the endotoxin to dry onto the base of the vial. This takes, in this author's experience, a surprisingly long time. The endotoxin should be applied to the base of the vial and the vials dried (so that there is no visible endotoxin) under a unidirectional airflow cabinet to protect the exposed vials from contamination. There will be a degree of loss through evaporation, although the extent of this is assessed through the recovery from the control vials. Typically, a 0.1 ml inoculum of endotoxin solution can take eight hours to dry onto the surface of the glass. In order to increase the recovery of endotoxin, Novitsky (23) recommends freeze-drying the challenge (rather than air-drying), although this requires specialized equipment that is not available to all laboratories. Other techniques include using an elevated temperature to increase the rate of drying. The method used to dry endotoxin should be validated, specifically to ensure that the method does not affect the challenge level of endotoxin.

Prior validation work should be conducted to determine the time needed between spiking the vials and placing them into the depyrogenation device (and at what temperature they are required to be stored at, with 2-8°C being typical). Work must also be carried out to determine the expiry time of vials that have passed through the depyrogenation device (i.e., how long can the vials remain before they are required to be tested. Typically, this time should not exceed 24 hours).

Once the depyrogenation cycle has been completed, the spiked containers or endotoxin vials are removed and tested using the LAL assay, and the remaining level of endotoxin is assessed. This is performed by adding a known level of pyrogen free water to the control and test vials. The amount of water should be sufficient to cover the base of the device and allow rinsing. The vials require different techniques in order to remove the endotoxin from the glass surface. These are typically variations of vortex mixing and ultrasonication. The actual times required for optimal endotoxin recovery will need to be assessed by the user. A dispersing agent or buffer may also be used in place of pyrogen-free water.

An aliquot is then tested against an endotoxin standard series (consisting of a minimum of three-log concentrations of endotoxin). The standard series should be prepared using the same lot of endotoxin used to challenge the vials.

The control vials require dilution prior to testing. The level of dilution will depend upon the expected level of endotoxin to be recovered. Negative control vials are also required. Normally, two types of negative controls are used. The first set is uninoculated vials, which are not put into the depyrogenation device. The result from these will indicate if any residual endotoxin is present. The second set consists of vials that have passed through the depyrogenation device. These are tested in the event that the elevated temperature inside the depyrogenation device has resulted in the leeching of any interfering substances that might inhibit the LAL assay. Both types of negative controls should show a low level of residual endotoxin of below the calculated three-logs. The negative control vials are tested in the same way as the spiked test vials. In order to claim depyrogenation, the device must show a three-log reduction of endotoxin of a 1000 EU or more challenge as per *USP*<1211>.

The following factors introduce variability into depyrogenation studies and may affect the success of the study:

- The material being challenged. For example, a glass vial behaves differently to an aluminum cap.
- For glass, the type of glass the challenge vials are made from (e.g., Type I or Type II glass. Type I glass is borosilicate characterized by a high degree of hydrolytic stability; Type II glass is soda-lime).
- The type of depyrogenation device and its efficiency. One key difference between dry heat ovens is the HEPA filter type and housing. Some filters

shed a high number of particles during temperature transition (i.e., the rate of temperature change). Generally, the faster the temperature increases the more particles that are generated. This phenomenon is also affected by degree of airflow uniformity and pressure balance. Some of these particles may contain endotoxin or interfere with the LAL assay (24).

- The method used to dry the endotoxin to the container being tested.
- The mechanism of the depyrogenation device. Akers found that devices that dry heat depyrogenate using infrared are more effective (25).
- Different manufacturers endotoxin varies based on the extent of natural or artificial contaminants. This is dependent upon how pure the endotoxin is (i.e., whether other cellular components are present) and whether the endotoxin contains fillers, such as glycol. These factors may increase or decrease the time taken to achieve heat inactivation.

CONCLUSION

This paper has examined a mechanism of depyrogenation using dry heat inactivation. Depyrogenation forms part of a critical process in many pharmaceutical production facilities, particularly where glass vials and bottles are required for aseptic filling operations. There remain some important questions relating to depyrogenation studies, such as the number of validation runs and frequency of re-validation. The number of validation runs is commonly set at three in order to demonstrate reproducibility, but this number is not fixed. The frequency of re-validation is to be determined by the user based on risk assessment, although annual re-qualification using endotoxin is not uncommon.

It should not be forgotten, even achieving successful depyrogenation, that the pyroburden that presents a risk to pharmaceuticals is derived from a combination of raw materials, water, active ingredients, environment, and primary packaging materials. Risks of endotoxin or other pyrogenic contamination can arise from multiple sources and not simply from the final containers alone.

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ARTICLE ACRONYM LISTING

BET	Bacterial Endotoxin Test
CSE	Control Standard Endotoxin
DEAE	Diethylaminoethyl Cellulose
EU	Endotoxin Units
HCl	Hydrochloric Acid
HEPA	High-Efficiency Particulate Air
LAL	Limulus Amebocyte Lysate
LPS	Lipopolysaccharide
NaOH	Sodium Hydroxide
RO	Reverse Osmosis
USP	United States Pharmacopeia
WFI	Water for Injection

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