A Comparative Study of
Different Methods for
Endotoxin Destruction

Introduction

Dry heat is the established method of depyrogenation within the pharmaceutical industry. This paper describes a series of studies undertaken to determine whether successful depyrogenation can be achieved practicably using methods other than dry heat. Depyrogenation refers to the removal or inactivation of pyrogens. In practice, depyrogenation processes are qualified by demonstrating that they are capable of reducing bacterial endotoxin to an acceptance level. Depyrogenation, like sterilization, is an absolute term that can only be theoretically demonstrated because of test insensitivity [1].

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. In laboratories, it is important that glassware used for bacterial endotoxin testing is depyrogenated to minimize contamination of the test; sampling containers also need to be free of pyrogens to avoid contamination of samples and the detection of false positives [2]. The main pyrogenic substance, which poses a risk to glassware used in the production of parenterals, is endotoxin. Endotoxin is the natural heat stable lipopolysaccharide contained in the outer walls of Gram-negative bacteria [3]. It is released into the environment during bacterial cell death, lysis, growth and multiplication [4, 5]. Endotoxin is considered to be the most significant pyrogen due to its ubiquity and potency [6].

One concern with endotoxin in relation to pharmaceuticals is that they are heat stable, making them resistant to most conventional sterilization processes and thus necessitating separate tests for viable cells and endotoxin. Pharmaceutical processes and equipment are at risk from endotoxin. Thus depyrogenation is an important factor in maintaining sterility assurance during the preparation of pharmaceutical products. There are several different means to achieve depyrogenation (including ultrafiltration; ion exchange chromatography and the use of acid-base hydrolysis). Arguably the most common depyrogenation devices are those which operate using dry-heat (such as a depyrogenation tunnel using unidirectional hot air, which is used to prepare primary packaging articles – product vials – for aseptic filling) [7].

Requirements for depyrogenation differ amongst the regulatory bodies. The European Pharmacopeia specifies dry heat at 250°C for 30 minutes, or 200°C for 60 minutes for depyrogenation of glass used for pyrogen tests, although there is no written requirement for the depyrogenation of glassware used for parenterals [8]. Glassware to be used for LAL testing must be depyrogenated to a level lower than the sensitivity of the test. The USP <1211> and FDA guidelines do not contain a temperature specification, but rather require a three-log reduction in endotoxin from a starting concentration of at least 1000 Endotoxin Units (EU) for depyrogenation [9]. In terms of the depyrogenation reaction, endotoxin, when dry heat inactivated, follows a linear log-reduction curve until reduction to three-logs. After this destruction continues to
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occur but does not necessarily follow linear regression, it is ‘bi-phasic’ reduction [10].

Endotoxin reduction is assessed using Endotoxin Indicators, which are prepared using a preparation of Control Standard Endotoxin (CSE) derived from *Escherichia coli* 0113:H10. The CSE used should be similar to endotoxin used to perform routine the Bacterial Endotoxin Test (BET) using *Limulus* Amoebocyte Lysate (LAL) methodology and traceable to a reference standard. There is some debate within the industry regarding how an Endotoxin Indicator is prepared; this is either by adding endotoxin to the surface of the item to be depyrogenated and drying it, or by using a pre-prepared endotoxin indicator. Of the two approaches, the first approach is the preferred by the author. This is because it is considered the greater challenge as the endotoxin is applied directly to the surface of the device to be depyrogenated [11].

**Study Design**

The aim of the study was to determine if successful depyrogenation could be achieved practically using methods other than dry heat. Two alternative methods were investigated: moist heat (autoclaving) and a caustic rinse, and the results compared with dry heat depyrogenation.

**Acceptance Criteria**

There is no compendial endotoxin tolerance limit (K) published for glass final product containers so the limit for medical devices was used in this study, which is 20 EU/device [12].

**Methodology**

The method selected for the study was the kinetic-turbidimetric LAL (*Limulus* amebocyte lysate) test (as per the Bacterial Endotoxins Test, European Pharmacopoeia <2.6.14>). The LAL test is based on the lysate enzyme isolated from the horseshoe crab, which clots in the presence of endotoxin [3]. In practice, for the kinetic-turbidimetric test, the LAL reaction rates are expressed in terms of the time taken to reach a predetermined ‘threshold’ optical density, known as the onset time [13]. Higher endotoxin concentrations result in shorter onset times. Endotoxin concentrations are calculated from a standard curve (*E. coli* endotoxin standard), constructed by linear regression of log onset time versus log endotoxin concentration [14]. Samples, standards and controls are tested at least in duplicate. Positive product control recovery should be in the range of 50 – 200%.

The study used glass vials that had been rinsed with Water-for-Injection. A 50, 000 EU/ml solution of endotoxin was prepared. Vials were inoculated with 0.1ml of the solution to give a theoretical 5, 000 EU spike and were air-dried in a unidirectional airflow cabinet overnight. A 0.1 ml inoculation volume was chosen because the literature reports that smaller volumes of inocula reduce adsorption leading to higher and more consistent endotoxin recoveries from the vials.

Once prepared, Endotoxin Indicators were placed in defined locations in a depyrogenation device (10 Endotoxin Indicators were considered sufficient to assess the depyrogenation capabilities), along with two positive controls.

**Treatment and Testing**

Three treatments were performed:

1. **Dry heat:** vials were treated in a Carbolite oven at 250°C for 30 minutes. Heating at 250°C for not less than 30 minutes to depyrogenate glassware and utensils is stated in USP Pyrogen Test Chapter <151> [3].

2. **Moist heat:** vials were autoclaved at 121°C for 30 minutes; this autoclave cycle is a standard one used to sterilize equipment.

3. **Caustic:** each vial was rinsed with 10ml of 0.1N NaOH for 1 minute. This concentration of caustic is reported in the literature as capable of depyrogenation [17]. A 1-minute wash was performed as it was practicable and could be easily implemented and carried out if shown to be successful.

Three runs were performed for each treatment, except for dry heat as it was shown that depyrogenation was 100% successful after the first two runs (as might be expected from an established and mature technology).

After treatment, each vial had 5 ml of buffer added and was sonicated in an ultrasonic bath for 15 minutes, then placed onto an orbital shaker until tested. All bottles were vortex mixed for one minute just before testing. A combination of ultrasonication plus vortexing is suggested in the literature to give optimal endotoxin recovery.

All samples were tested in duplicate against a standard curve ranging from 5.0 EU/ml to 0.005 EU/ml. Positive controls, post moist heat treatment samples and post caustic treatment samples required dilution so that detectable endotoxin was within the range of the standard curve. For each run at least one treated sample and all controls were also tested spiked with 0.5 EU/ml of endotoxin. To test the samples, a 1:2 dilution with LAL reagent water was performed in the lysate reaction tube (for samples and controls requiring dilution to fall within the range of the standard curve, this additional 1:2 dilution was performed as part of testing). For the positive product control the 1:2 dilution was performed with a 1.0 EU/ml endotoxin standard solution resulting in a dilution the same as the test sample but with a 0.5 EU/ml endotoxin spike. Positive product control spike recoveries between 50-200% indicate the absence of interference, and the suitability of dilutions for testing.

**Results**

**Data Analysis**

Data analysis was performed using Microsoft Excel™ and Systat 11™. A two-way ANOVA was performed to test for significant differences between the three treatments. Differences were confirmed using Student’s t-test. As the post treatment endotoxin concentrations measured are dependent on the initial spike for each run, statistical analysis was performed on the log endotoxin reduction, rather than the actual endotoxin measured. This approach reduces the risk of detecting false significances and does not assume that the data is from the same population.

<table>
<thead>
<tr>
<th>Log endotoxin reduction</th>
<th>Dry Heat Treatment</th>
<th>Moist Heat Treatment</th>
<th>Caustic Treatment</th>
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</thead>
<tbody>
<tr>
<td><strong>Mean</strong></td>
<td>5.2</td>
<td>1.7</td>
<td>1.7</td>
</tr>
<tr>
<td><strong>Maximum</strong></td>
<td>5.4</td>
<td>2.5</td>
<td>2.9</td>
</tr>
<tr>
<td><strong>Minimum</strong></td>
<td>5.0</td>
<td>1.0</td>
<td>1.0</td>
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Statistical analysis indicated that dry heat was the most effective treatment for depyrogenation, reducing endotoxin significantly more...
than both the moist heat and caustic treatments. Whilst dry heat treatment was the most effective method, the mean log endotoxin reduction after both moist and caustic treatment was 1.7 and thus there was no significant difference between these two depyrogenation methods.

**Discussion**

This study examined the depyrogenation effectiveness of three different treatments on glassware. The results indicated that dry heat treatment consistently depyrogenated all of the spiked bottles with a greater than 3-log reduction in endotoxin. This was expected as dry heat is a generally accepted method of depyrogenation. This is notwithstanding that the mechanism of endotoxin destruction by dry heat not having been definitively studied, although it is probably due to indiscriminate incineration of molecules due to the very high temperatures. Ludwig and Avis [15] suggest a free radical mediated oxidation reaction, catalyzed by trace metals from the manufacturing process.

With the moist heat method it was demonstrated in this study that a conventional sterilizing autoclave cycle was considerably less effective in reducing endotoxin concentrations than dry heat. The 3-log depyrogenation target was not achieved for any of the moist heat treated bottles, and the post-treatment endotoxin levels in all of the test bottles were above 4 EU/ml. Endotoxin is renowned for its thermostability, and moist heat treatment by conventional autoclaving is ineffective for depyrogenation. As a way forward, successful depyrogenation by autoclaving in the presence of hydrogen peroxide has been reported, destruction in this case is thought due to the oxidation of the fatty acids in the lipid A portion of the lipopolysaccharide [16]. Furthermore, autoclaving for longer periods has also been shown to depyrogenate successfully. These are avenues that could be explored in future studies; however neither approach is practicable for the typical laboratory.

Treatment of spiked bottles with sodium hydroxide demonstrated a negative effect on endotoxin; however the 3-log depyrogenation target could not be consistently achieved. Whilst the endotoxin in a number of bottles was reduced to a level below the acceptance limit, this was inconsistent. It should be noted that the caustic wash was performed by hand; hence the inconsistencies in depyrogenation may be linked to the variability of mixing. The mechanism of destruction during caustic treatment is due to the hydrolysis of ester and amide linkages found in the lipid A portion of endotoxin. The alkaline hydrolysis of ester linkages resulting in an alcohol and acid salt is called saponification and can be enhanced by heating [17]. Therefore a greater reduction in endotoxin may have been achieved by including a heating stage in the caustic treatment.

**Conclusion**

Dry heat was determined to be the most effective method of depyrogenation performing significantly better than both the moist heat and caustic treatments. This study therefore supported the general findings in most literature. Dry heat consistently achieved a greater than 3-log endotoxin reduction. The moist heat and caustic treatment protocols in this study were not capable of consistent depyrogenation. Neither did the two alternative methods achieve the same quantifiable level of endotoxin destruction compared to dry heat.

The vials used in this study were rinsed with Water for Injection before treatment. Purchased glass bottles often contain impurities and need rinsing. Recycled glassware may have chemical and biological deposits that could distort or mask endotoxin residues present. These impurities and residues may affect the depyrogenation of the glass and should be removed prior to treatment; there is thus scope for the development of procedures to prepare glassware for depyrogenation. Undertaking measures to reduce endotoxin is an important part of microbiological control, in relation to pharmaceutical processing and for laboratories required to prepare materials or to test components. This relates to the risk of endotoxin. The pathological effects of endotoxin, when injected, are a rapid increase in core body temperature followed by extremely rapid and severe shock, often followed by death before the cause is even diagnosed [18].

**References**