LAL UPDATE®

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Dear LAL User,

This issue of the LAL UPDATE returns to a subject we have covered in several previous issues of this publication (LAL UPDATE Vol.4, No.1, Vol.6 No.2, and Vol.8, No.1). The current article by Dr. Michael E. Dawson addresses the validation of depyrogenation in more detail than the earlier ones.

As Associates of Cape Cod, Inc. enters its 20th year in the LAL business and the LAL UPDATE gets ready to celebrate its 11th birthday, users should expect to see some changes in the newsletter. With the next publication of the LAL UPDATE another publication, the LAL UPDATE International Edition will make its debut. The two publications will be identical except that the International Edition will also include international market issues, such as changes in the European or Japanese Pharmacopeias. The calendar section and new product service announcements will also be more territory-specific. For those of our readers who need to follow LAL regulations abroad, I suggest you write us for a subscription to both issues. We also hope to publish the International Edition in Spanish, German and French.

Sincerely,

Thomas J. Novitsky, Ph.D.
Editor

Depyrogenation

by Michael E. Dawson, Ph.D.

Depyrogenation is the removal or destruction of pyrogens, and particularly endotoxin. Endotoxin removal from an article is validated by showing a multi-log reduction in the endotoxin load from an initial concentration. Gram-negative bacterial endotoxin is the most significant pyrogen in most situations and is also the most refractory. Since conditions required to destroy endotoxin will destroy other pyrogens, the term depyrogenation is appropriate.

Depyrogenation can be accomplished in a number of ways, but thermal destruction of endotoxin is the most common and effective method for heat stable articles. This LAL UPDATE addresses the question of depyrogenation by dry heat and its validation.

In order to set up a depyrogenation procedure, an appropriate temperature and exposure time must be determined. Temperatures in excess of 180°C effectively destroy endotoxin. Below 180°C, depyrogenation may be incomplete even after extended periods. The time required to achieve a given level of endotoxin destruction decreases as temperature increases, e.g., a three log reduction requires 65.4 minutes at 190°C but only 1.5 minutes at 250°C (Tsuji and Lewis, 1978). Unfortunately, different rates of endotoxin destruction have been found in different studies. For example, based on results from three studies, Avis et al. (1987) cite times of 10, 130 and 500 minutes for equivalent destruction of endotoxin at 250°C. The USP BET states "such as 250°C or above for sufficient time" and references the informational chapter <1211> "Sterilization and Sterility Assurance of Compendial Articles".

Depyrogenation studies have adopted concepts and terminology from sterilization and an excellent practical monograph published by the PDA deals with both topics. (Validation of Dry Heat Processes Used for Sterilization and Depyrogenation, PDA Technical Report No. 3, PDA, Philadelphia, PA). Three parameters appropriated from the theory of sterilization that are frequently encountered in the discussions of depyrogenation are D value, z value and F value.

***D* value**: the time required to give a one log (90%) destruction of endotoxin at a given temperature - thus a time of $3D$ will give a three log reduction.

***z* value**: the temperature increment required to change the D value by one log. It requires the D value to be known at two temperatures and assumes linear kinetics for the destruction of endotoxin.

***F* value**: the time required to give equivalent destruction at different temperatures. An F value is specific for a stated degree of destruction, for example, two log or three logs. F values are calculated using a reference temperature and a z value.

These parameters are useful for theoretical discussions of endotoxin destruction and particularly for comparisons made within a single study. However, significant differences between the measured parameters are evident when studies are compared. Ludwig and Avis discuss marked discrepancies between F values in different studies. These authors suggest that a primary cause for the discrepancies is differences in the control of the sample temperature, the rate of heating and rates of introduction and removal of sample. Another difference can be the type of vessel being depyrogenated (endotoxin carrier). These authors have also demonstrated clearly that different endotoxin preparations (particularly those with fillers) show markedly different rates of depyrogenation. Finally, Tsuji and Harrison (1987) report that the destruction of endotoxin does not follow the simple logarithmic decline exhibited in sterilization studies on spore suspensions. Hecker and coworkers (1993) reiterate this point and state that two D values are required to describe endotoxin destruction. If it is not possible to determine a D value that is valid for the whole curve, z and F values are similarly uncertain. These authors also recommend that endotoxin preparations with fillers should not be used for depyrogenation studies because of their relative ease of destruction.

The discrepancies surrounding published values for these parameters provide little guidance for selection of a temperature/time regime for depyrogenation. However, Tsuji and Lewis (1978), Ludwig and Avis (1990), and Hecker et al. (1993) are in general agreement that a three log destruction of endotoxin is accomplished in two minutes or less at 250° C. Thus, the "classical regime" of 250°C for 30 minutes offers a high level of assurance of at least a three log reduction. This regime also has the advantage of precedent, having been widely used in the industry for many years.

In order to ensure depyrogenation, it is important to note that every article in the oven must be exposed to at least the stated temperature for no less than the stated time. The total time over which the oven is operating will always be longer than the time stated at temperature to allow for the coldest part of the load to heat up.

Validation of Dry Heat Depyrogenation Processes

Before validation can begin, the process temperature and time must be selected. It is common to add a safety factor of either time or temperature. At least three hours at a minimum of 180°C or 30 minutes at 250°C are commonly used. Consider the type of material to be depyrogenated. For example, silicon tubing can be effectively depyrogenated by dry heat, but temperatures of 250°C make it brittle and prone to split or break. Extremes of time and temperature should be avoided. An hour at a process temperature of 250°C is reasonable. Caution: excessive periods at high temperatures are at best a waste of energy and may actually damage materials being depyrogenated.

An important consideration is whether the process is being validated for all types of articles that might be introduced into the oven, or whether depyrogenation of a particular article is being validated. Generally the process is validated for all types of articles. It is therefore important to perform the validation using worst case conditions, which usually translates to the greatest possible load. However, it may be necessary to validate a second time/temperature regime for particular articles, such as the silicon tubing mentioned above.

Validation of endotoxin destruction under the selected conditions requires two phases. First, the physical heating characteristics of the oven must be established and cold spots identified. Then, the proposed cycle must be challenged with endotoxin. At least a three log reduction of endotoxin must be demonstrated.

Phase 1

Use calibrated temperature probes (thermistors or thermocouples) in vials/vessels throughout the oven under maximum load. Include probes in the middle of the load. A common minimum probe configuration is an X pattern of five probes on the top, middle and bottom shelves. Additional probes may be necessary in large ovens while fewer may be justified for small ones. Use calibrated multichannel recorders to record the data. Run the oven to identify the cold spots and to determine the time required to reach temperature at that point in the load. It may be necessary to try different loads to determine the one with the slowest heating to ensure that the validation is conducted for the worst case. Determine the run time and temperature to ensure that the process temperature is maintained for the required time, with a safety margin.

Depyrogenation that takes place during heat up and cool down is not considered when the process time is selected, thus adding a safety factor of time. Also, the oven temperature may be set above the selected process temperature in order to reduce the time for the coldest part of the load to reach the required temperature. In this case, the temperature in the oven will continue to rise above the stated process temperature until equilibrium is reached, adding a safety factor of temperature.

The advantage of setting the oven temperature higher than that required for depyrogenation is illustrated in Figures 1 and 2. For example, with the oven temperature set to 250°C, the cold spot does not reach the process temperature of 250°C for more than one and a half hours (Figure 1). With the oven temperature set at 300°C the cold spot in the load reaches temperature in less than 1 hour (Figure 2).

Phase 2

Prepare or obtain challenge articles to demonstrate that the process time and temperature are sufficient to effect at least a three log destruction of endotoxin. These can be prepared by adding a small volume of a high endotoxin concentration to give at least 1000 EU (stated in USP chapter <1211>) to challenge articles. Sufficient endotoxin should be added to give 1000 EU recoverable. If 10% is the lowest acceptable recovery, an addition of 10,000 EU is appropriate.

Associates of Cape Cod, Inc.'s high potency control standard endotoxin (CSE catalog number 800-3) is intended for use in depyrogenation studies. Each vial

contains 125µg endotoxin, or 25 µg/ml when the contents are reconstituted in 5 ml. Assuming a potency of 10 EU/ng, this is equivalent to 250,000 EU/ml. Thus 40µl will contain 10,000 EU and can be used to spike the challenge articles. Dry the added endotoxin on the articles (air dry or lyophilize). Suitable articles include glass vials or ampoules. A vessel that allows a recovery volume of 1 ml is convenient. Test the recovery of added endotoxin. Recovery of at least 10% of the added endotoxin is desirable. If depyrogenation of large vessels is to be validated, challenge vials can be placed inside the large vessel.

A paper published by the LAL Users' Group (1989) discusses the preparation of endotoxin indicators (challenge articles) from vials that can then be used in depyrogenation studies. Using amber glass makes it easy to find the challenge vials in a load. The authors note that if challenge articles are to be stored for later use, it should be demonstrated that the articles do not lose potency during storage.

Alternatively, vials of lyophilized endotoxin of an appropriate concentration can be used directly as challenge articles. Associates of Cape Cod, Inc.'s 0.5µg endotoxin/vial, (catalog number 800-1) can be used directly as challenge articles provided that stoppers and labels are removed. This is a convenient way to perform depyrogenation studies and avoids the problem of poor recovery of endotoxin. Associates of Cape Cod, Inc.'s control standard endotoxins are registered as medical devices under a 510K submission for use in depyrogenation validation.

Distribute challenge articles throughout the oven, including the cold spot, and include them as part of a full load. Once again, five vials per shelf in an X pattern, on at least the top, middle and bottom shelves, is a common arrangement. Additional vials should be used at the cold spots if these are not already covered by the other vials. At least six vials should be left out of the oven as untreated controls. If the challenge article used is a well defined product with little vial to vial variability, it may not be necessary to test as many as six control vials.

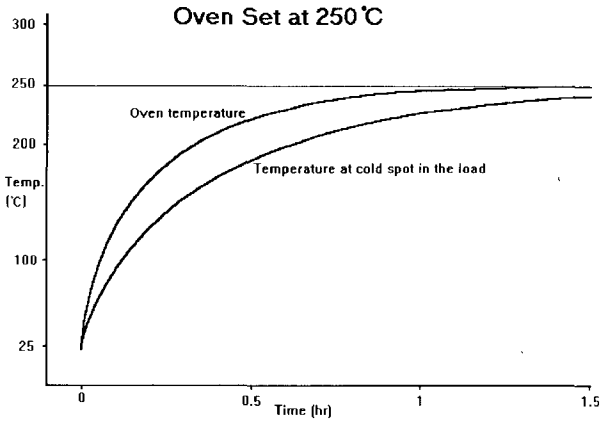

Figure 1. Equilibration of oven thermostat and cold spot of an oven at 250°C

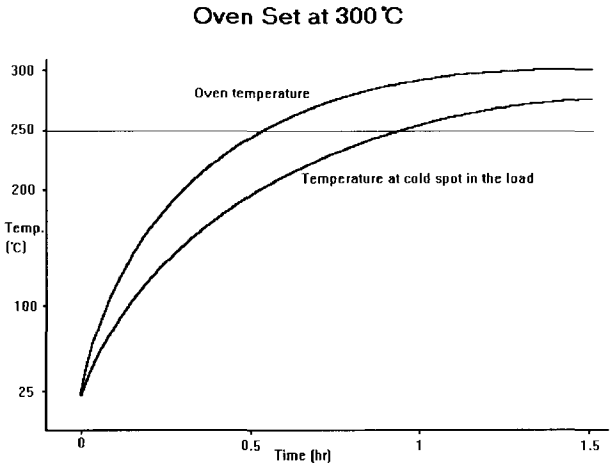

Figure 2. Equilibration of oven thermostat and cold spot of an oven at 300°C

If recoveries are very variable, a larger number should be tested.

Recover endotoxin from the untreated and heat-treated articles by adding a known volume of LAL reagent water (LRW). A volume of 1 ml is convenient and simplifies calculations. Endotoxin recovery can be effected by vigorous vortexing or sonication. Usually vortexing in the same way as is used to reconstitute a vial of CSE is sufficient, i.e., vortex for one minute initially and then every 10 minutes for 30-60 minutes. If recovery is poor e.g., less than 20%, the period of vortexing can be extended or sonication can be attempted. Novitsky et al. (1986) reported on the effectiveness of recovery of endotoxin from different surfaces by vortexing and sonication.

Perform an LAL test on the extracts. Ensure that the test or reagent is sensitive enough to detect at least a three log reduction from the measured endotoxin concentration in the untreated vial extract. The endotoxin detected in the treated article extract should be at least three logs less than that in the extract from the untreated controls. A four log reduction is recommended. If the gel-clot method is used, an endpoint test can be used such that, if the extract tests negative, at least a three log reduction has been demonstrated. Only if a positive result is obtained, is it necessary to test a series of dilutions to determine whether at least a three log reduction has been obtained. Include positive product controls for the processed articles to control for the possibility that negative results are due to inhibition rather than absence of endotoxin.

For example:

If 10 ml vials are used as challenge articles and are spiked with 40µl of high potency endotoxin, the nominal endotoxin challenge is 10,000 EU, assuming that the potency of the endotoxin is 10 EU/ng with the LAL lot being used. If endotoxin is recovered by adding 1 ml LRW to the article, then EU/ml is equivalent to EU/article. If the mean measured concentration of the untreated controls is 5,000 EU/ml, the recovery rate is 50%.

It is recommended that the extracts from treated articles (processed in the depyrogenation cycle) be tested in a limits test. If they show no detectable endotoxin, this represents destruction by a factor of at least 5000 EU/ml ÷ 0.03 (125) EU/ml =160,000 or > 5 log reduction. The extract from the treated articles should be tested both spiked and unspiked in the normal way to ensure that negative results are due to absence of endotoxin and not inhibition.

If the treated extracts test positive in the limits test it is still possible that at least a three log destruction of endotoxin has been achieved and extracts should be assayed. Provided that the endpoints are all obtained at extract dilutions of less than 1:160, destruction of endotoxin is at least three logs for all articles. No single article should have an endpoint at 1:160 for the depyrogenation process to be considered valid. Note: twofold dilutions from 1:2.5 will include 1:160 in the dilution scheme.

Write up the report, file it and have it available for inspection. The SOP should state the conditions that will necessitate revalidation. If the physical data (temperature and time) assures that specified minimum conditions are met or exceeded, it should not be necessary to repeat the endotoxin challenge study (Phase II). However, an annual test using a challenge article placed at the cold spot is a reasonable check. Be sure the temperature probes are calibrated regularly and that the original "worst case" loading condition is not exceeded.

Each individual oven must be validated. With some thought this method can be adapted to tunnel ovens and the same principles may be applied to stopper washers.

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Calendar

The Center for Professional Advancement
"LAL Testing: Medical Devices and Biotechnology"
Amsterdam April 25-27, 1994
Course Director
Michael E. Dawson, Ph.D.
Assistant Director, ACC

National Association of Nephrology Technologists
Marriott Hotel
LaGuardia Airport
May 16, 1994
"Endotoxin Contamination in Hemodialysis Systems"
by Marilyn J. Gould, Ph.D.
Associate Director, ACC

94th American Society for Microbiology
May 23-27, 1994
Las Vegas Convention Center
Visit Associates of Cape Cod, Inc.
at Booth #1153

IBC's Fourth Annual Conference
The Shift in R&D Strategies for the Prevention & Treatment of Endotoxemia & Sepsis
The Ritz-Carlton, Philadelphia, PA
"Limulus Endotoxin Neutralizing Protein: Potential for Human Therapy"
by Thomas J. Novitsky, Ph.D.,
VP/Director, ACC
June 13-15, 1994

Biomedical Focus VIII Conference and Exposition
Minneapolis Convention Center
Minneapolis, MN
July 25-27, 1994
ACC Tabletop Exhibit

International Endotoxin Society Third Conference
Marina Congress Center
Helsinki, Finland
August 15-18, 1994