LAL Update®

ASSOCIATES OF CAPE COD INCORPORATED JUNE 1997 / VOL. 15, NO. 2

Letter from the President

The Limulus amebocyte collection season at ACC is now in full swing. Fortunately there are no signs of population decreases for our Cape Cod horseshoe crabs as have been seen in Delaware Bay. We continue to be vigilant however and are involved in a number of studies and conservation efforts. Dr. Dawson, who oversees ACC's horseshoe crab collection/return activities, is an invertebrate physiologist by training and has recently been appointed as a member of the Horseshoe Crab Subcommittee of the American Eel Advisory Panel, a part of the Atlantic States Marine Fisheries Commission. He is also active with local groups where the welfare of the horseshoe crab is concerned.

My own work in marine ecology and life-long interest in ornithology also provides a network of contacts concerned about horseshoe crabs. In May I visited Japan and the People's Republic of China. Although China seems to have a sufficient supply of horseshoe crabs (Tachypleus sp.) to meet their own growing demand, Japan's crab population is extremely small and may not withstand further taking for any reason. It is not surprising, therefore, that ACC exports LAL to Japan. In China we are exploring ways to help their lysate industry come up to US standards, but have no delusions that Tachypleus lysate will make up any future shortfall of LAL in the USA or European markets.

ACC's long-term goal is to find a synthetic replacement for LAL while finding ways to reduce LAL usage and protect existing stocks of horseshoe crabs. In the meantime, we are maintaining a sufficient supply of both raw lysate and finished product to ensure our customers an uninterrupted supply of reagent.

On a different note, this issue of the UPDATE discusses routine testing and retesting for all LAL methods. Have a good summer!

Sincerely,

Thomas J. Novitsky, Ph.D.

Routine Testing and Retests

Introduction

This LAL Update addresses routine testing, the next logical step following the articles in the two previous issues (1,2), which addressed inhibition and enhancement testing for validation of LAL assays. It also ends a series of occasional articles that have addressed fundamentals of LAL testing, including endotoxin limits (3), maximum valid dilution and minimum valid concentration (4), preliminary testing (5) and inhibition/enhancement testing. This article addresses the specifics of routine testing of finished product for the various LAL methods. It includes the provisions for retests of test failures from different regulatory documents. While the article does not specifically address in-process testing, the same controls and considerations apply. Some issues are common to all LAL test methods while others are method specific. These are discussed in turn.

General Considerations

For parenteral drugs and biological products, the FDA guideline (6) states that "The sampling technique selected and the number of units to be tested should be based on the manufacturing procedures and the batch size. A minimum of three units, representing the beginning, the middle and end, should be tested from a lot." Contents of vials/containers may be pooled for testing. If vials are pooled, the endotoxin limit (and thus the MVD) should be divided by the number of vials pooled. This corrects for the possibility of contamination or an interfering factor in one container being diluted out when pooled with the contents of uncontaminated containers. This issue was discussed

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in more detail in the LAL Update article on Preliminary Testing (5). It is not addressed in any of the regulatory documents. There is no requirement to make a correction for pooling medical device extracts (see the LAL Update (7)). The FDA guideline does not give any specific information about sampling of medical device lots. However, a sampling scheme is given in the section on inhibition and enhancement testing, which was covered in the LAL Update article on this topic (1). Routine testing should be carried out following the same procedure used for inhibition or enhancement testing; therefore the same sampling plan should be used. All of the regulatory documents agree that endotoxin standards, negative controls, samples and positive product controls should be tested in at least duplicate, no matter which LAL test method is used.

Technical Notice

Disposables used in LAL applications must be tested before use to show that they are not contaminated with endotoxin, do not adsorb endotoxin, and do not have extractable substances that interfere with the test. We were surprised recently to have to reject a lot of polystyrene test tubes obtained from a manufacturer whose product has always been satisfactory. Pleases contact our Technical Services Dept. if you experience difficulties with disposables.

The Gel-clot Method

The requirements for the gel-clot method will depend upon which regulatory documents are drawn on for the development of the standard operating procedure (SOP). Regarding endotoxin standards, if an SOP states that the USP Bacterial Endotoxins Test (8) procedure will be followed, then a series of endotoxin standards should be included with every test. The FDA guideline states that, provided that consistency has been demonstrated in the test laboratory, standards do not have to be included with every test. It states that a series of endotoxin standards must be included in the first test of the day and that a 2λ positive control and a negative control must be included with each subsequent test. For medical devices, the FDA guideline states that standards should be included with routine tests but it later states that they may be omitted once consistency of standard endpoints has been demonstrated. The guideline also states that "a standard series should be run when confirming end-product contamination," that is, when performing retests. In the European Pharmacopoeia (EP) Bacterial Endotoxins chapter (9), a standard series is not required with routine tests, only a negative control and 2λ positive control. While this may be acceptable to European authorities, this is not recommended for product that will be sold in the United States.

The product itself should be tested in duplicate at the dilution at which the test was validated. Positive product controls (sometimes abbreviated to PPCs), consisting of product spiked with endotoxin to contain a 2λ concentration (that is, twice the label claim sensitivity of the lysate), must be included in parallel with the unspiked product. The positive product control serves as an inhibition control for the gel-clot method.

Interpretation of test results is very important and should be described in the SOP for routine testing. The procedure should make a clear distinction between an invalid test and a test failure. An invalid test is not a test failure, but clearly product cannot be released based on an invalid test. In order for a test to be valid the following conditions must be met:

1. *The negative controls must test negative*
2. *The geometric mean endpoint of the standard series must be within a factor of two of the label claim sensitivity of the LAL reagent, or, in the absence of a series, the positive controls must test positive*
3. *The positive product controls (spiked sample) must test positive*

If these conditions are satisfied, the results for the unspiked product can be considered valid. If a valid test is negative at a product dilution not exceeding the maximum valid dilution (MVD), then the product contains less than the endotoxin limit and passes the test (see LAL Update, 13(4) for a detailed discussion of MVDs).

A positive result in a valid test at a dilution less than the MVD does not necessarily mean that the product exceeds the endotoxin limit and fails the test. Only when a positive result has been obtained at the MVD does the product fail at the endotoxin limit. For example, consider a product with an endotoxin limit of 12.5 EU/ml. Assume that the labeled sensitivity of the Pyrotell (λ) used is 0.125 EU/ml, so the MVD is 100. If the product is tested at 1:25 dilution and gives a valid positive result, the endotoxin concentration is at least 25 x 0.125 EU/ml = 3.1 EU/ml. We cannot give a precise value for the endotoxin concentration because we have not tested a series of dilutions and obtained an endpoint (the greatest dilution at which the product tests positive). So we do not know

whether the endotoxin concentration is greater than 12.5 EU/ml or not. A second test must be performed on a series of dilutions including the MVD (or on the MVD alone). Provided that the product tests negative at the MVD, it contains less than 12.5 EU/ml and passes the test. This second test at the MVD is not a retest of product that exceeds the endotoxin limit because the initial test did not indicate a failure.

Endpoint Methods

For both endpoint and kinetic methods, the routine test procedure is similar to the inhibition and enhancement test for methods validation. As with the gel-clot test, the FDA guideline states that endotoxin standards should be included in the first test of the day. In subsequent tests, only a 4λ positive control and a negative control need to be included. For both endpoint and kinetic tests, λ is the lowest concentration on the standard curve, which is the detection limit of the assay and so is equivalent to the label claim sensitivity in the gel-clot test. When standard series is not included in a test, data analysis is performed using the earlier standard curve, provided that the endotoxin concentration for the positive control is determined within +/- 25% of the actual (i.e. nominal) concentration. The guideline also states that standards should be included when performing retests or if the alternate procedure is used, in which standards are diluted in product containing no detectable endotoxin (see LAL Update, 5(2)). Despite the provision in the guideline, it is recommended that a standard series be included with all tests. A positive product control should be included for each product or sample tested. Positive product controls consist of product at the test concentration and contain standard endotoxin added at a concentration of 4λ.

To pass the test, the test must be valid. This requires that negative controls contain no detectable endotoxin; the correlation coefficient of the standard curve should be at least 0.980; and positive product control must be recovered within +/- 25% after subtraction of any endotoxin detected in the unspiked product. If a positive control is used in place of a standard series, it must also be recovered within the same limits.

Over the last 10 years there has been some discussion about what the spike recovery "within +/ 25%" should refer to. It has been generally agreed that this is relative to the *measured* endotoxin concentration of the 4λ endotoxin standard in water (or positive control). This being so, it is reasonable to require that the measured endotoxin concentration of the 4λ endotoxin standard in water be within 25% of the nominal 4λ concentration, as is the case for positive controls. Note that this is not stated in any of the regulatory documents. So, if the known (nominal) 4λ concentration is 0.10 EU/ml and the measured concentration is 0.12 EU/ml, a spike recovery of 0.14 EU/ml in the product would pass the +/- 25% spike recovery requirement.

Provided the test is valid, the product passes if it is shown to contain less endotoxin than the endotoxin limit. If no endotoxin is detected, it should be reported to contain a concentration less than that of the lowest endotoxin standard multiplied by the dilution of the product sample.

Kinetic Methods

The provisions in the FDA guideline for the kinetic test are similar to those for endpoint methods but there are some important differences, particularly when the FDA guidance (10) is considered. The FDA guideline and guidance state that it is not necessary to include a series of endotoxin standards and the use of an "archived standard curve" is permissible as long as "consistency of standards curves has been

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JULY

July 24 - July 26
LAL Methodology and Applications Seminar and Workshop
Minneapolis, MN

July 28 - July 29
Biomedical Focus Conference
Minneapolis Convention Center
Minneapolis, MN

SEPTEMBER

September 15 - September 17
LAL Methodology and Applications Seminar and Workshop
Baltimore, MD

September 30 - October 2
LAL Methodology and Applications Seminar and Workshop
Falmouth, MA

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demonstrated in the test laboratory." The guidance continues to state that consistency is determined "by regression analysis of the data points from the standard curves generated over three consecutive test days (minimum of three curves). If the coefficient of correlation, r, meets the criteria in Appendix A then consistency is proved and the curve becomes the 'archived curve.' " Appendix A simply requires that the absolute value of r be at least 0.980. Archived standard curves are an attractive idea but it is our experience that for most users, they are more trouble than they are worth. If archived curves are used, positive controls with an endotoxin concentration from the middle of the standard curve must be included in assays without a full series of standards. The positive control must be quantified within +/ 25% of the nominal standard endotoxin concentration. Despite these provisions for omitting standard curves, as with endpoint methods, we recommend that standards be included with every assay.

Product should be tested unspiked and spiked, just as it was tested for inhibition or enhancement, and at the same dilution. The required spike recovery for a valid assay is +/- 50% after subtraction of any endotoxin detected in the unspiked product; this is the same as for validation. Negative controls should contain less endotoxin than the lowest standard concentration. To pass the test, the sample of product must be shown to contain less endotoxin than the endotoxin limit in a valid assay.

Retests

The USP BET does not mention retests. However, the USP Transfusion and Infusion Assemblies chapter (11) does allow for one retest of medical device extracts by the BET. The FDA guideline allows for two retests of failures.

According to the FDA guideline, the first retest is to insure that the test itself was not contaminated. It is a repeat test of the original sample of product (pooled or not), but with double the original number of replicates. The second retest is to check for the possibility that the sample(s) of the article were contaminated after they had been taken. It consists of taking 10 new units of product and testing them individually. All units must pass the test. In the guideline section on medical devices, it is not stated that extracts must be tested individually. For the gel-clot method, product should be tested at the MVD.

In the light of the Barr decision (12), investigation of a test failure, which is clearly an out-of-specification (OOS) result, is required and the failure must be explained before the batch of product can be passed on the basis of a retest. Invalid test results should also be subject to an investigation.

The EP, which describes the gel-clot method and states that tests should be conducted at the MVD, only allows for retests if one replicate tests positive and the other negative.

There are three points mentioned above that relate to retests which are worth repeating. First, the inclusion of standards is required when retesting. Second, a repeat of an invalid test should not be counted as a retest of a product test failure. Finally, a product has not failed by the gel-clot method (i.e., shown to contain endotoxin equal to or greater than the endotoxin limit) until it tests positive at the MVD.

Conclusion

Perhaps the most important requirement for routine release testing of product is that the test must be valid. For all of the test methods the procedure is straightforward, and, for the endpoint and kinetic methods, it is the same as for the inhibition or enhancement test. Provided that in-process testing has been conducted to assure that product components are free of significant concentrations of endotoxin and that manufacturing processes are properly controlled, the release test should become a necessary formality.

References

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2. *Inhibition or Enhancement Testing: Part 2. LAL Update 15(1), March, 1997.*
3. *Endotoxin Limits. LAL Update 13(2), June, 1995.*
4. *Maximum Valid Dilution and Minimum Valid Concentration, LAL Update, 13(4), December, 1995.*
5. *Preliminary Testing. LAL Update 14(1), March, 1996.*
6. *"Guideline on Validation of the Limulus Amebocyte Lysate Test as an End-Product Endotoxin Test for Human and Animal Parenteral Drugs, Biological Products and Medical Devices." U.S. Department of Health and Human Services, Public Health Service, Food and Drug Administration, December, 1987.*
7. *USP Changes Medical Devices Chapter. LAL Update 12(4), December, 1994.*
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12. *United States of America, Plaintiff, v. Barr Laboratories, Inc. et al., Defendants. 1993. United States District Court for the District of New Jersey. Opinion Rendered by Alfred M. Wolin, U.S.D.J.*