LAL UPDATE®

Volume 11, No. 2 **May 1993**

Dear LAL User,

This issue of the LAL UPDATE reviews the latest revision to the USP BET. The revisions to the BET have been debated for some time now and on May 15, 1993, the revised chapter is official.

The revision well illustrates the USP process. Three in-process revisions were published along with comments before a consensus was reached. Although a lot of credit for the revisions goes to Terry Munson, the LAL Users Group, the LAL manufacturers, and LAL users in general, the USP should be commended for a fine effort. The final product is not only understandable, it is actually quite reasonable.

Significant changes which affect the pocketbook are the reduction of the number of vials of CSE needed for a standardization with RSE from 4 to 1, and likewise a reduction of the number of vials of LAL needed for a confirmation of labeled sensitivity from 4 to 1. These changes should also save some operator time. Besides some clarification, (especially in the sections on Inhibition/Enhancement, Geometric Mean Calculation, and Endotoxin Content Calculation) the RSE reconstitution procedure (timing) has been changed as well as the pH range for an optimal LAL assay (from 6.0 - 7.5 to 6.0 - 8.0).

As always, we encourage our readers to participate in the USP review process by reading and commenting on in-process revisions which appear in the Pharmacopeial Forum. For my part, the LAL UPDATE will continue to alert LAL users on USP articles involving LAL and the Pyrogen test through the USP Says column.

Sincerely,

Thomas J. Novitsky, Ph.D.
Editor

<85> Bacterial Endotoxins Test
(8th Supplement to USP XXII)

Change to read:

This chapter provides a test for estimating the concentration of bacterial endotoxins that may be present in or on the sample of the article(s) to which the test is applied using Limulus Amebocyte Lysate (LAL) which has been obtained from aqueous extracts of the circulating amebocytes of the horseshoe crab, *Limulus polyphemus*, and which has been prepared and characterized for use as an LAL reagent for gel-clot formation.

The determination of the reaction endpoint is made with dilutions from the material under test in direct comparison with parallel dilutions of a reference endotoxin, and quantities of endotoxin are expressed in defined Endotoxin Units.

Since LAL reagents have also been formulated to be used for turbidimetric or colorimetric readings, such tests may be used if shown to comply with the requirements for alternative methods. These tests require the establishment of a standard regression curve and the endotoxin content of the test material is determined by interpolation from the curve.The procedures include incubation for a preselected time of reacting endotoxin and control solutions with LAL reagent and reading of the spectrophotometric light absorbance at suitable wavelengths. In the case of the endpoint turbidimetric procedure, the reading is made immediately at the end of the incubation period. In the kinetic assays (turbidimetric and colorimetric),the absorbance is measured throughout the reaction period and rate values are determined from those readings. In the endpoint colormetric procedure the reaction is arrested at the end of the preselected time by the addition of an enzyme-reaction-terminating agent prior to the readings.

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REFERENCE STANDARD AND CONTROL STANDARD ENDOTOXINS

Change to read:

The reference standard endotoxin (RSE) is the USP Endotoxin Reference Standard, which has a defined potency of 10,000 USP Endotoxin Units (EU) per vial. Constitute the entire contents of 1 vial of the RSE with 5 mL of LAL Reagent Water [1], mix intermittently for 30 minutes, using a vortex mixer, and use this concentrate for making appropriate serial dilutions. Preserve the concentrate in a refrigerator, for making subsequent dilutions, for not more than 14 days. Mix vigorously, using a vortex mixer, for not less than 3 minutes before use. Mix each dilution for not less than 30 seconds before proceeding to make the next dilution. Do not store dilutions, because of loss of activity by adsorption, in the absence of supporting data to the contrary. A control standard endotoxin (CSE) is an endotoxin preparation other than the RSE that has been standardized against the RSE. Standardize each new lot of CSE prior to use in the test. Calibration of a CSE in terms of the RSE must be with the specific lot of LAL reagent and the test procedure with which it is to be used. Standardization of a CSE against the RSE using an LAL reagent for the gel-clot procedure may be effected by assaying a minimum of 1 vial of the CSE and 1 vial of the RSE, as directed under *Test Procedure*, but using 4 replicate reaction tubes at each level of the dilution series of the RSE and 4 replicate reaction tubes similarly for each vial or aliquot of the CSE. The antilog of the difference between the mean $\log_{10}$ endpoint of the RSE and the mean $\log_{10}$ endpoint of the CSE is the standardized potency of the CSE, which then is to be converted to and expressed in Endotoxin Units per ng under stated drying conditions for the CSE, or in Endotoxin Units per container, whichever is appropriate.

A suitable CSE has a potency of not less than 2 Endotoxin units per ng and not more than 50 Endotoxin Units per ng.

PREPARATORY TESTING

Change to read:

Use an LAL reagent of confirmed label sensitivity. Treat any containers or utensils employed so as to destroy extraneous surface endotoxins that may be present, such as by heating in oven at 250° or above for sufficient time[2].

The validity of test results for bacterial endotoxins requires an adequate demonstration that specimens of the article, or of solutions, washings, or extracts thereof to which the test is to be applied do not of themselves inhibit or enhance the reaction or otherwise interfere with the test. Validation is accomplished by performing the *Inhibition or Enhancement Test* as described below. Appropriate negative controls are included. Validation must be repeated if the LAL reagent source or the method of manufacture or formulation of the article is changed.

Change to read:

Test for Confirmation of Labeled LAL Reagent Sensitivity

Confirm the labeled sensitivity using at least one vial of the LAL reagent lot. Prepare a series of two-fold dilutions of the RSE (or CSE) to give concentrations of 2λ,λ, 0.5λ, and 0.25λ, where λ is the labeled sensitivity of the LAL reagent in Endotoxin Units per mL. Perform the test on the four standard concentrations in quadruplicate and include negative controls. The geometric mean endpoint concentration (see *Calculations and Interpretation*) must be greater than or equal to 0.5λ and less than or equal to 2.0λ. Confirm the labeled sensitivity of each new lot of LAL reagent prior to use in the test.

Change to read:

Inhibition or Enhancement Test

Perform the test on aliquots of the specimen, or a dilution not to exceed the *Maximum Valid Dilution,* in which there is no detectable endotoxin. Perform the test on the specimen without added endotoxin and with endotoxin added to give final concentrations of 2.0λ ,λ, 0.5λ and 0.25λ. Perform the test as directed under *Test Procedure*, but using not less than 4 replicate tubes for the untreated specimen and for each specimen to which endotoxin has been added. In parallel with the above, test in duplicate the same endotoxin concentrations in water and untreated negative controls. Calculate the geometric mean endpoint endotoxin concentration for the specimen as described under *Calculations and Interpretation*. The test is valid for the article if the geometric mean endpoint concentration in the specimen is greater than or equal to 0.5λ and less than or equal to 2.0λ.

If the result obtained for the specimens to which endotoxin has been added is outside the specified limit, the article is unsuitable for the *Bacterial Endotoxins Test.*

Repeat the test for inhibition or enhancement after neutralization, inactivation, or removal of the interfering

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[1] LAL-Reagent Water-Sterile Water for Injection or other water that shows no reaction with the specific LAL Reagent with which it is to be used, at the limit of sensitivity of such reagent.

[2] For a test for validity of procedure for inactivation of endotoxins, see "Dry-heat Sterilization" under *Sterilization and Sterility Assurance of Compendial Articles* <1211>. Use a LAL Reagent having a sensitivity of not less than 0.15 Endotoxin Units per mL.

substances or after the specimen has been diluted by a factor not exceeding the *Maximum Valid Dilution*. Use a dilution, not to exceed the *Maximum Valid Dilution,* sufficient to overcome the inhibition or enhancement of the known added endotoxin, for subsequent assays of endotoxin in test specimens.

If endogeneous endotoxin is detectable in the untreated specimens under the conditions of the test, the article is unsuitable for the *Inhibition or Enhancement Test*, or, it may be rendered suitable by removing the endotoxin present by ultra-filtration, or by appropriate dilution. Dilute the untreated specimen (as constituted, where applicable, for administration or use), to a level not exceeding the maximum valid dilution, at which no endotoxin is detectable. Repeat the test for *Inhibition or Enhancement* using the specimens at those dilutions.

Add the following:

Maximum Valid Dilution (MVD)

The Maximum Valid Dilution is appropriate to Injections or to solutions for parenteral administration in the form constituted or diluted for administration, or where applicable, to the amount of drug by weight if the volume of the dosage form for administration could be varied. Where the endotoxin limit concentration is specified in the individual monograph in terms of volume (in EU per mL), divide the limit by λ, which is the labeled sensitivity (in EU per mL) of the lysate employed in the assay, to obtain the MVD factor. Where the endotoxin limit concentration is specified in the individual monograph in terms of weight or of Units of active drug (in EU per mg or in EU per Unit), multiply the limit by the concentration (in mg per mL or in Units per mL) of the drug in the solution tested or of the drug constituted according to the label instructions, whichever is applicable, and divide the product of the multiplication by λ, to obtain the MVD factor. The MVD factor so obtained is the limit dilution factor for the preparation for the test to be valid.

TEST PROCEDURE

Change to read:

In preparing for and applying the test, observe precautions in handling the specimens in order to avoid gross microbial contamination. To quantify the amount of endotoxin in a specimen, an assay is performed on decreasing concentrations of specimens prepared by serial dilution. Select dilutions so that they correspond to a geometric series in which each step is greater than the next by a constant ratio. Include negative and positive controls, and a positive product control.

Use not less than 2 replicate reaction tubes at each level of the dilution series for each specimen under test. A standard endotoxin dilution series involving not less than 2 replicate reaction tubes is conducted in parallel. A set of standard endotoxin dilution series is included for each block of tubes, which may consist of a number of racks for incubation together, provided the environmental conditions within blocks are uniform.

Change to read:

Preparation

Since the form and amount per container of standard endotoxin and of LAL reagent may vary, constitution and/or dilution of contents should be as directed in the labeling. The pH of the test mixture of the specimen and the LAL reagent is in the range 6.0 to 8.0 unless specifically directed otherwise in the individual monograph. The pH may be adjusted by the addition of sterile, endotoxin-free sodium hydroxide or hydrochloric acid or suitable buffers to the specimen prior to testing.

Change to read:

Procedure

Into 10- X 75-mm test tubes or single test vials, dispense the specified volumes of negative controls, standard endotoxin concentrations, specimens, and positive product controls. Positive product controls consist of the article, or of solution or washing extract thereof to which RSE, or a standardized CSE, has been added to give a concentration of 2λ. Add appropriately constituted LAL reagent, unless single test vials are used. Mix the specimen/LAL reagent mixture and place in an incubating device such as a water bath or heating block, accurately recording the time at which the tubes are so placed. Incubate each tube, undisturbed, for 60 ±2 minutes at 37 ±1°, and carefully remove it for observation. A positive reaction is characterized by the formation of a firm gel that remains when inverted through 180°. Record such a result as positive (+). A negative result is characterized by the absence of such a gel or by the formation of a viscous gel that does not maintain its integrity. Record such a result as negative (-). Handle the tubes with care, and avoid subjecting them to unwanted vibrations, or false negative observations may result. The test is invalid if the positive product control is negative or the endotoxin standard does not show the endpoint concentration to be within ±1 two-fold dilutions from the label claim sensitivity of the LAL reagent or if any negative control is positive. Proceed to *Endotoxin Content Calculation* to determine the amount of endotoxin present in the test specimen.

CALCULATION AND INTERPRETATION

Add the following:

Geometric Mean Calculation

The endpoint is the last positive test in a series of decreasing concentrations of endotoxin, specimen, or specimen to which endotoxin has been added. Record the endpoint concentration, E, for each replicate series of dilutions. Determine the log end-point concentrations, e, and calculate the geometric mean endpoint concentration using the following formula:

Geometric Mean Endpoint Concentration = antilog $(\Sigma e/f)$, where Σe is the sum of the log endpoint concentrations of the dilution series used and f is the number of replicates.

Change to read:

Endotoxin Content Calculation

Calculate the concentration of endotoxin (in Units per mL or in Units per g or mg) in or on the article under test. First, calculate the endpoint concentration, E, for each of a series of dilutions by multiplying the reciprocal of each endpoint dilution factor by λ, where λ is the labeled sensitivity expressed in Endotoxin units per mL of the lysate used in the test. The geometric endpoint concentration of the article under test is thus the antilog of $\Sigma e/f$, where e is the log of the endpoint concentration, and f is the number of replicate reaction tubes read at the level for the specimen under test.

Change to read:

Interpretation

The article meets the requirements of the test if the concentration of endotoxin is not more than that specified in the individual monograph.

Calendar

The Third Annual Meeting on Advances in the Diagnosis, Prevention and Treatment of Endotoxemia & Sepsis
June 17-18, 1993
The Ritz-Carlton
Philadelphia, PA
"A New Chromogenic *Limulus* Amebocyte Lysate Kit for the Detection of Endotoxin in Human Blood"
by Thomas J. Novitsky, Ph.D., VP/Director

International Conference on Endotoxin
Associates of Cape Cod, Inc. - Co-Sponsors
Academic Medical Centre
Amsterdam, The Netherlands
August 17, 1993
Wet Workshop-Visit our booth

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