LAL UPDATE®

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Dear LAL User,

This UPDATE will readdress the issue of endotoxin standards, which continues to annoy LAL users. Currently, there are five "official" worldwide endotoxin standards: EC-5 (US-FDA), lot F (US-USP), International Reference Preparation (Europe-EP), International Standard for Endotoxin (Europe-WHO), and UTK-B (Japan-JP). In addition, there are any number of Control Standard Endotoxins, or CSE's. For example, each manufacturer of LAL has at least one CSE, and some have several. Since the majority of LAL manufacturers are US-based and are regulated by the USFDA under CFR 21, there can only be one "official" standard or Reference Standard Endotoxin (RSE) and that is EC-5 (note: lot F sold by the USP is exactly the same as EC-5). Therefore, the other "official" standards are really only CSE's when LAL released with an EC-5 sensitivity is used. While in the United States, the use of a European standard may only affect some multinational corporations, the routine use of a variety of CSE's does add a layer of complexity for all LAL users.

It is likely that EC-5 will be replaced soon by EC-6. Hopefully the problems encountered when EC-5 replaced EC-2 will not be repeated. Perhaps this is also an opportunity for the USP or WHO to take a leadership position and produce a truly international standard, one provided to all LAL users, government agencies and other pharmacopeias at a reasonable cost. If used for all required in-process, end product release, and compendial LAL tests, a CSE would not be required and LAL tests would be controlled to the highest degree.

The following article, "Endotoxin Standards and CSE Potency", will address the endotoxin problem in detail.

Sincerely,

Thomas J. Novitsky, Ph.D.
Editor

Endotoxin Standards and CSE Potency

by Michael E. Dawson, Ph.D.

In the early years of the LAL test a variety of different endotoxin preparations were used as standards. Results of endotoxin assays were, and sometimes still are, expressed in nanograms of endotoxin per milliliter (ng/ml, 1 ng = 10^{-9}g). Expressing amounts of endotoxin in units of weight is not very useful because the biological activity of endotoxins varies greatly between gram negative bacterial species and between different strains of the same species. Activity may be tested as pyrogenicity (*i.e.* the ability of the endotoxin to induce fever) or LAL reactivity. Various assays take advantage of the stimulatory effect of endotoxin upon the immune system, such as macrophage activation assays. Very different pyrogenicities (in man and rabbits) of endotoxins from different organisms have been reported by Greisman and Hornick (1).

Threshold Pyrogenic Doses of Different Endotoxin Preparations

Endotoxin	Threshold Pyrogenic Dose (ng/kg)
Salmonella typhosa	0.1 - 0.14
Escherichia coli	1.0
Pseudomonas sp.	50 - 70

(data from Greisman and Hornick)

Marked differences in pyrogenicity and LAL reactivity were demonstrated in a study of eleven different lipopolysaccharide (LPS) preparations (2). There are other reports of differences in rabbit pyrogenicity and activity in the LAL test (3,4,5) between endotoxins. These studies demonstrated an imperfect correlation between the pyrogenicity and LAL activity. Galanos *et al* (6) described how the chemical composition of an LPS preparation and its biological activity are influenced by the growth conditions of the organism, the extraction procedure and the degree of purification. Non-LPS components influence the aggregation state of the endotoxin and its activity. Significant differences in potency have even been found in different lots of endotoxin prepared from the same bulk extract (7).

Consequently, results reported in nanograms cannot reliably be compared to one another because a nanogram of endotoxin preparation A may have a quite different potency from that of endotoxin B. Therefore, endotoxin limits cannot be expressed in units of weight (usually nanograms) without a clear specification of which standard endotoxin preparation is being used. Also, reporting results in nanograms can lead to a misunderstanding of their meaning. The endotoxin used as a standard is usually not the same as that present in the sample. An LAL test result reported in units of weight seems to imply that a certain mass of endotoxin is present. The result actually means that the endotoxin concentration in the sample had an activity equivalent to that of the stated mass of standard endotoxin. This distinction may not be important if relative masses (or activities) of endotoxin are being compared within a particular study, but it is a distinction that is frequently not appreciated.

The Reference Standard Endotoxin and the Endotoxin Unit

The FDA recognized the problems of different potencies with different endotoxin preparations and concluded that a suitable endotoxin preparation was necessary to standardize the LAL test. A large bulk preparation of *Escherichia coli* (Braude strain) O113:H10K negative endotoxin was prepared by Westphal extraction (8), characterized and designated EC.

Hochstein (9) describes how the first small pilot lot of standard endotoxin was prepared from this stock in 1976 and was designated EC-1. EC-2 was a larger batch of vials containing 1.0 µg of endotoxin which like EC-1, was lyophilized with 0.1% Normal Serum Albumin (human). After accumulating a large amount of data and experience with this lot, a potency (or activity) of 5000 Endotoxin Units per vial (or 5 EU/ng) was assigned to it. One Endotoxin Unit (EU) was defined as 0.2 ng of EC-2 and was an expression of the activity of the endotoxin in an LAL assay. This was an important step because it avoided the problem of the different activities per unit weight for different endotoxin preparations. Having defined the endotoxin unit, it was necessary to produce a sufficiently large batch of standard endotoxin for use by the pharmaceutical industry and other LAL users. After two attempts at producing a standard (EC-3 and EC-4), the FDA contracted with Mallinckrodt to prepare a large batch of standard endotoxin. Specifications for the lot of reference standard endotoxin (RSE) were drawn up by the (then) FDA Office of Biologics and the USP. Problems were solved and the formulation was modified in pilot runs. Bulk EC material was dried under vacuum at 50°C and dissolved in a solution of 2% lactose and 2% polyethylene glycol 6000 prior to filling and lyophilization.

Approximately 30,000 vials of EC-5 were produced and the uniformity of the lot was verified. The potency was determined relative to EC-2 and found to be 2.1 times greater than that of the earlier standard. This was rounded to 2 to give 10,000 EU/vial (7). The endotoxin was divided between the Office of Biologics (now the Center for Biologics Evaluation and Research, CBER) and the USP. The USP refers to the preparation as the endotoxin reference standard, lot F, and this can be purchased

from the USP. There was a discrepancy between the reconstitution procedures for EC-5 and lot F but this has been remedied in the revision to the USP Bacterial Endotoxins Test Chapter (10).

An important consequence of the development of the RSE was that all licensed manufacturers were required to label their (gel-clot) LAL reagent with a sensitivity determined with lot EC-5 obtained from the Office of Biologics. Thus, for the first time a primary standard was widely available and the sensitivities of LAL lots from different manufacturers were comparable (11). Another advantage was that reporting results in EU only gives information about the activity of the endotoxin detected, without creating a misleading impression that the absolute amount of endotoxin is being quantified. The use of endotoxin units also allows for the potencies of a secondary or control standard endotoxin (CSE) to be expressed in EU per nanogram of CSE. This is important because the RSE is expensive to purchase, only has a two week shelf-life after reconstitution and is limited in supply.

The International Standard

Following an international collaborative study in 1981-82, the WHO Expert Committee on Biological Standardization concluded that international standardization of endotoxin detection with LAL reagents had not yet been achieved (5). Since that time EC-5/USP lot F has been produced and made available to the WHO (7). The WHO, however, decided to produce an international standard and obtained some of the same bulk EC endotoxin (8) from which EC-5 was prepared. The endotoxin was dispensed in 1 ml aliquots into approximately 4000 ampules at a concentration of 2 μg/ml in a 0.3% (w/v) trehalose solution. The standard was lyophilized, secondarily desiccated and the ampules were sealed under dry nitrogen and stored at -20° C in the dark (5). An international collaborative study was conducted and the potency of this preparation was determined to be 14,000 International Units (IU) per ampule. Thus the potency of the endotoxin preparation is 7 IU/ng. One IU was set to equal one EU.

In equating the IU to the EU, it was intended that the International Standard could serve as a direct substitute for EC-5 and avoid the need for EU/IU conversions. However, it is well known that the potency of one endotoxin relative to another is influenced by the LAL reagent with which it is determined (3,4). For this reason the USP Bacterial Endotoxins Test (BET) chapter states "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" (10). Thus, in the eyes of the USP, a potency is only valid for a specific LAL lot. The BET defines a CSE as "an endotoxin preparation other than the RSE that has been standardized against the RSE" and the International Standard clearly fills this description.

This discussion is largely academic in the case of quantitative LAL test methods in which a standard curve is prepared and endotoxin concentrations are determined with reference to the curve. If the standard concentrations are expressed in IU (or any other units), assay results for test samples are in the same units. The problem arises with the gel-clot test, the test method described by the chapters on endotoxin testing in the USP, the EP and other pharmacopeia. Gel-clot LAL reagents supplied by manufacturers licensed by the FDA CBER have a labelled sensitivity determined in EU/ml using EC-5. Like the USP BET chapter, the EP Endotoxins Test chapter requires confirmation of the LAL reagent label claim and it refers to the International Standard as the primary endotoxin standard (12). If the average potency of the International Standard (relative to the RSE) does not apply for the LAL lot being used, then it will not be possible to confirm the labelled sensitivity. This problem is well illustrated by the collaborative study results themselves (5). Of 123 determinations of potency by the gel-clot test for the (then proposed) International Standard, 21 (17%) were more than a factor of two greater or less than the mean potency. Two estimates were about 6 times greater than the mean; one was 6 times less than the mean and another was 22 times less! In another study in which the gel-clot method was used, the potency of the International Standard was 0.7 IU/EU for the LAL lot used (13). This is not significantly different from the original 1 IU/EU determination given the accepted plus or minus a factor of two error for the gel-clot test. While the mean potency is clearly applicable in the majority of cases, problems with the confirmation of label claim can be expected to occur with significant frequency. There is anecdotal evidence of such problems, but none that has been reported in the literature.

A similar situation has occurred in Japan where a national primary endotoxin standard has been established (14). The standard is an *E. coli* UTK-B preparation. Like the International Standard, one Japanese Endotoxin Unit (EU) has been set to be equivalent to 1 EU of EC-5. With some lots of LAL reagent, users have been unable to confirm the labelled sensitivity that had been determined with EC-5.

Because both of these endotoxin preparations are referenced back to EC-5, they can be considered secondary standards, particularly when they are used in conjunction with an LAL reagent with a sensitivity determined using EC-5. If the labelled sensitivity cannot be confirmed with a particular LAL lot, the potency of the standard can be determined with reference to the RSE as described in the USP. Because the RSE is used to do this, the procedure will indicate whether there is truly a potency discrepancy or some other problem with the test or reagents.

The European Standard

In Europe, a secondary standard has been adopted by the European Pharmacopeia. This standard was prepared from a highly purified *Salmonella abortus equi* (15) and ampuled in liquid form by Pyroquant Diagnostik GmbH, Walldorf, Germany. Lot NP-2 was adopted by the European Pharmacopeia (EP) as the Biological

Reference Preparation (BRP) in the Endotoxins Test chapter (12). The EP specified that the potency of NP-2 be determined relative to the International Standard. Thus the NP-2 was the official secondary standard or control standard endotoxin (CSE) in the EP. Unfortunately, NP-2 was found to be contaminated. While freshly opened ampules were of the stated potency, upon storage and exposure to oxygen, microbial growth occurred and the potency of the standard increased. Lot NP-3 was introduced as a replacement in 1992 and referred to as the 2nd European Endotoxin Reference Preparation (16). In contrast with NP-2, which was labelled in ng/ml, NP-3 is supplied in ampules with a labelled potency of 800 International Units (IU) per milliliter and is sterile.

The decision to give a potency in IU has potential for causing problems for LAL users. In the discussion of the International Standard it was noted that by assigning a mean potency, it would sometimes be difficult or impossible to confirm label claim in the gel-clot method. The European standard has a single potency assigned and so is two steps removed from the primary standard, EC-5, further compounding the potential for problems in the confirmation of label claim.

Control Standard Endotoxins and their Potency

Control standard endotoxins are generally considered to be endotoxins other than the reference standards, the USP definition notwithstanding. Practically speaking, CSE's are those provided by LAL manufacturers for use with LAL reagents. Provision is made in the USP and in the FDA Guideline (17) for the use of CSE.

The fact that CSE potency varies when determined with different LAL lots has been considered above. Consequently, certificates of analysis supplied by Associates of Cape Cod show the potency of the CSE determined relative to the RSE (EC-5) **using a specific lot of LAL reagent**. In the gel-clot method, potency of a CSE is determined by testing parallel series of dilutions of RSE and the CSE (expressed in ng/ml). The geometric mean endpoint of the RSE (which must be within a factor of two of the labelled sensitivity) is divided by the geometric mean endpoint of the CSE. The result is the potency of the CSE expressed in EU/ng. The USP BET chapter (10) specifies a procedure for the determination of CSE potency.

For the chromogenic and turbidimetric methods, a standard curve is constructed using dilutions of RSE. A series of known concentrations of CSE (in ng/ml) is assayed and the results expressed in EU/ml. For each CSE dilution with a mean measured endotoxin concentration (in EU/ml) falling within the range of the RSE standard curve, the measured endotoxin concentration is divided by known concentration (in ng/ml). The potency for the vial of CSE is the mean of the potencies of the individual CSE concentrations. The calculated potency only applies for the LAL lot with which it was determined. Procedures for the determination of CSE potency by quantitative methods are given in Appendix C of the FDA Guideline (17).

The fact that a single lot of CSE can exhibit potencies with different LAL lots can be disconcerting to those new to LAL testing. This might be due in part to real differences between LAL regents and their relative sensitivities to different endotoxin preparations (e.g. RSE and a CSE). In the gel-clot method, twofold dilutions of sample and standard are tested and the resolution of the method is limited to the twofold increments. Thus, if the potency of a CSE is determined by gel-clot to be 10 EU/ng, this does not mean that is the actual or true potency. If it could be determined, the actual potency might be 7 EU/ng, but the method cannot discriminate between the twofold dilutions (geometric mean endpoints not withstanding).

Compounding the problem is the fact that the labelled sensitivities of gel-clot reagents are themselves determined using a twofold series of endotoxin dilutions. A labelled sensitivity of 0.125 EU/ml means that this reagent clotted at 0.125 EU/ml but not at 0.0625 EU/ml, so the labelled sensitivity is an approximation. The error associated with this method is therefore plus or minus one twofold dilution. This is accepted by regulatory agencies as well as LAL users and also applied to the potency of a CSE.

Consequently, potencies of 5 EU/ng for LAL lot A and 10 EU/ng for lot B determined for the same CSE are not significantly different. Each is the best approximation of the potency for that particular LAL lot. However, it is important to use the value for a particular combination for LAL and CSE lots. Not doing so can result in failure to confirm labelled sensitivity and invalid tests.

There is less published information about potencies determined by the quantitative chromogenic and turbidimetric methods. Case and Novitsky (18) report that relative endotoxin potency varies with LAL lot in a kinetic turbidimetric assay. Poole and Mussett report significant variability between and within laboratories using chromogenic and turbidimetric methods in the multi-center study in which the potency of the International Standard was determined (5). The mean potencies of three endotoxin preparations (relative to the RSE) tended to be somewhat higher than those determined by the gel-clot test and to be rather less than those determined by rabbit pyrogen test. Interestingly, none of the potencies of the three endotoxin preparations studied were distributed normally about the mean, as it appeared to be in the gel-clot test. This could simply be due to small number of determinations made by the chromogenic and turbidimetric methods.

Conclusion

Control standard endotoxins are an economical alternative to the RSE and **are accepted by the FDA.** Certificates of Analysis **are also accepted**. A check on the validity of the potency is provided by the requirement to confirm label claim at least once a day when conducting gel-clot tests. If the potency is incorrect or is not valid, confirmation

of label claim will be difficult or impossible. It is therefore very important that the correct potency is applied. Just because the potency of an endotoxin lot was 10 EU/ng for the last three lots of LAL, it does not mean that it will still be 10 EU/ml for the next lot, even if the CSE lot is unchanged. It is clearly important that the concept of potency be understood by LAL users, particularly if they are using a CSE.

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"UPDATE-ING THE RECORD"

In a Special Edition of the "LAL Review" published by BioWhittaker (September, 1993) it was pointed out that the last LAL UPDATE® (Vol. 11, No. 3) contained "inaccuracies" relating to BioWhittaker's chromogenic products. Indeed, the name Kinetic QCL-1000 was inadvertently used instead of Kinetic-QCL® in reference to BioWhittaker's kinetic chromogenic LAL. I regret any confusion this may have caused.

We object to the implication that our assessment of Pyrochrome was dishonest. However, rather than argue semantics, we would like to let Pyrochrome speak for itself. If you are currently using a chromogenic LAL, call us for a sample of Pyrochrome and decide for yourself.

Editor