Variability and Test Error with the LAL Assay

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Introduction

The *Limulus* amebocyte lysate (LAL) assay is the compendial test for the examination of bacterial endotoxin in pharmaceutical products (as described in USP chapter <85>), in-process material, and pharmaceutical grade water.

With any biological tests, measurements are susceptible to variations in analytical conditions. Here the LAL assay has a relatively high level of variability even for a biological assay.[1] This variation derives from 3 principle sources: reagents, product, and method.[2] This paper examines some of the reasons for LAL test variation, focusing on photometric methods (chromogenic and turbidimetric), and considers how variation can be assessed through good laboratory quality control.

LAL Test Variability

Different aspects of the LAL test can cause variability. These include the LAL reagent, endotoxin control standard, standard curves, and dilution error. These are examined in turn.

LAL Reagent

The LAL reagent is a contributor to assay variation for the following reasons:

- The LAL reagent (lysate of the horseshoe crab *Limulus polyphemus*) is of biological origin. It is a complex mixture of enzymes and co-factors. The extract is relatively crude mixture and is not a single purified enzyme. This means that the enzyme activity cannot be determined exactly for each lot of lysate manufactured.
- The manufacturing process includes the addition of buffers and detergents which contribute a further source of variability.
- The enzymatic activity of each lot of LAL is assessed by the manufacturer using Reference Standard Endotoxin (RSE, supplied by the USP). The LAL sensitivity is assessed by performing a 2-fold dilution series. The RSE used to characterize the lysate is not readily available to all test laboratories because of its rarity and cost. Laboratories normally use Control Standard Endotoxin (CSE). The potency of CSE is determined by the lysate supplier assessing the CSE against RSE. This adds a potential area for test variation.

Bacterial Endotoxin

The endotoxin used in the assay can cause variation. This is because:

- The endotoxin used to prepare the CSE used in laboratories is from a purified strain of *Escherichia coli*. CSE is presented as a highly purified lipopolysaccharide free of most

detectable contaminants (such as proteins). The CSE contains additional stabilizing fillers like starch, human serum albumin, and polyethylene glycol. However, environmental endotoxin is not purified and normally takes the form of a macromolecular complex of lipopolysaccharide, cellular membrane proteins, and phospholipids which are shed by Gram-negative bacteria during growth and death. Thus there is variation in assaying environmental endotoxin against purified endotoxin standards.[3]

In addition, although the LAL test is specific for endotoxin, it will detect only the Lipid A portion of the endotoxin molecule which is available to activate the lysate (the activation of the clotting cascade, the Factor C pathway, is described below).[4] The Lipid-A portion of the endotoxin molecule may form aggregates which are not fully dispersed and therefore not homogenous enough to allow for accurate total measurement.

Thus, a sample which detects endotoxin may not show all of the endotoxin in the sample, for this depends upon the amount of Lipid-A available. Therefore, samples which detect endotoxin may be underestimates. Furthermore, a sample which detects endotoxin may not demonstrate the same level of endotoxin when repeated because the availability of Lipid-A may alter as the chemical nature and stability of the sample changes over time.

- It should also be noted that the toxicity and reactivity of different types of environmental endotoxin differs depending on the biological activity of the Lipid-A molecule for different bacterial species.

LAL Test Variability

The LAL assay has an inherent variability of 50% to 200% (or one 2-fold error either side of each endotoxin standard). Variation arises, for kinetic assays, from the slope of the endotoxin standard curve.[5]

Test variation can arise from a range of test inputs, including:

- Test tubes
- Disposable pipette tips
- Micropipette tips
  - For the above, plastics used in the performance of the BET (eg, microtiter plates, plastic dilution tubes) are often not made specifically for endotoxin testing
- Aseptic technique
- Assay technique
- Variations in pipetting
- Variations in preparing control standards
- Variations in preparing dilutions (which is magnified if the error occurs with the first dilution in the series)
  - Dilutions stored over the longer term will show change. Variable factors include temperature, vessel composition, dilution range, and volume of the dilution)
- Cross contamination
- Product or sample interference
- Sampling containers

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- Sample storage times and temperatures
- LAL instrument/module variability–different instruments may give different results;
- Presence of endotoxin in product (where endotoxin molecules behave differently or where the availability of Lipid-A varies)
- Addition of buffers to stabilize pH
  - Ancillary solutions may not be free of endotoxin.
  - Some of the above relate directly to the practices of the testing technician (such as preparing dilutions, pipetting, weighing raw materials, and aseptic technique)

Endotoxin Concentrations

The significance of error also increases as the endotoxin concentrations used for a standard series become smaller. For example, with a standard curve of 1.0 to 0.1 EU / mL errors of 50% to 200% will have a lesser impact than a standard series of 5.0 to 0.005 EU/mL, based on the smaller value of the last endotoxin concentration in the standard series. It is perhaps for these reasons the acceptable spike recovery of test controls listed in the pharmacopeia is 50% to 200%.

Dilution Errors

Errors can occur with test dilutions, especially with those relating to the dilution of endotoxin and creating a standard curve. To avoid the possibility of dilution error arising from the construction of the standard curve, it is recommended that the following controls are put in place:

- The dilution series should be qualified before each lot of endotoxin or lysate is released for routine use, by 3 technicians who verify the dilution series 3 times each. A similar exercise is undertaken for each new technician who is trained in the test.
- For routine assays, the dilution series should not vary between tests (that is, the same types of dilutions are always undertaken).
- The starting concentration of endotoxin should always begin with the same value (this is normally with 1000 EU/mL). Endotoxin standard curves are constructed from the same starting concentration of endotoxin: 1000 EU/mL. This is

> **A dilution error can still occur where a mistake is made on the part of the technician conducting the test.**

verified by reviewing the manufacture's Certificate of Analysis and from undertaking confirmatory tests of the manufacture's certificate by comparing the in-use Control Standard Endotoxin against Reference Standard Endotoxin (both a purified extract of *E. coli* O113:H10).

Nonetheless, with the above in place, a dilution error can still occur where a mistake is made on the part of the technician conducting the test.

Standard Curve Linearity

Standard curve consistency is an important feature of the LAL test. A change of only 1% in y-intercept for a linear standard curve can result in a 30% to 35% change in endotoxin determination. Hence a sample with a known 10 EU/mL can read 13.5 EU/mL, not because of a change in the endotoxin content of the sample, but because of a shift in the y-intercept. An important means to control variability in the turbidimetric LAL test is to keep an eye on the onset (reaction) times. Seemingly small changes in these onset times result in changes to linearity, slope, and y-intercept that can have a significant effect on the test result.[6]

Assessing Variation

Whilst steps can be taken to reduce variation, the principles of good quality control dictate that a laboratory should have an oversight and a means of assessing if tests are satisfactory and whether variation has been sufficiently excessive as to cause concern. One means of doing so is through reviewing the coefficient of variation (CV).

Applying the CV

CV is a measure of precision. The precision of an analytical procedure is the degree of agreement among individual test results (or, in assay terminology, the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of a single, homogenous volume of the biological matrix).[7] The CV is the standard deviation expressed as a percentage of the mean. As the mean between different samples increases, then the CV can be used to measure the variability.

Commonly, CV is expressed as a percentage between test replicates (%CV). This can be applied to standard curve points and to test sample replicates. With LAL, the common requirement is for the CV to be ≤10% or ≤25% (depending on the requirement set by the lysate vendor). The lower the %CV, the closer the level of precision between the different test replicates is (the 'scatter' from the mean is relatively small).

There are different approaches to the calculation of CVs. These relate to different ways of calculating the standard deviation. Once the approach for calculating the standard deviation has been adopted, the CV becomes expressed as the ratio between the standard deviation and the mean.

From this, the %CV can be calculated thus:

100% x (standard deviation/arithmetic mean)

From the above it can be seen that the CV is converted into a percentage by multiplying the obtained number by 100 to produce the %CV.[8]

In assessing LAL tests, %CV values calculated on measurements of EU/mL typically increase for lower concentrations of endotoxin, in that values of <10% can often be obtained for higher concentrations of endotoxin, whereas values of between 10% and 30% are obtained for lower values of endotoxin (typically towards the end of the standard curve and close to the limit of detection).

It should be noted at this point that different LAL suppliers have different acceptance criteria for the LAL test and calculate their %CVs, through software packages, in different ways. Some, for example, calculate the CV based on the results of obtained in Endotoxin Units per millilitre (EU/mL); whereas others calculate coefficient of variations based on the sample onset times (in milli-absorbance units).[9,10]

Detecting Dilution Errors

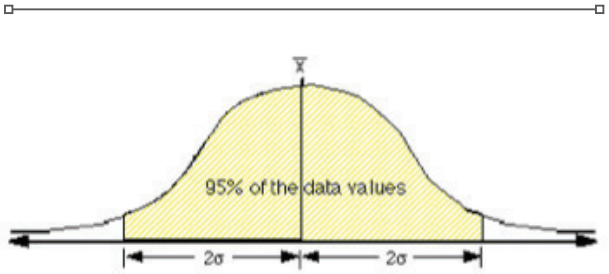
In addition to the coefficient of variation, dilution errors also require assessment. In order to check that the LAL assay has been performed correctly, the reaction time for the highest endotoxin concentration point on the standard curve should be examined to ensure that it lies within an expected time range in seconds. This is important because seemingly small changes in these onset times result in changes to linearity, slope, and y-intercept that can have a significant effect on the test result.[11]

For this, the onset times for the starting endotoxin concentration are examined (such as 5.0 EU/ml). This requires a study of historical data in order to establish the typical range. For greater accuracy, it is recommended that the first 100 tests performed using the endotoxin are assessed. If an error occurs with the preparation of the dilutions, then the onset time would fall outside of the expected range.

Examining the onset time is an important indicator of assay error as it directly relates to the way in which the photometric LAL test method works. With the kinetic-turbidimetric or chromogenic LAL test method, lysate (when aliquoted into reaction tubes) reacts with any endotoxin present in an aliquoted sample or within a standard curve dilution.[12] The reaction which takes place is one of turbidity or color change as measured against time. The faster the time taken to reach a turbidity threshold (measured in milli-absorbance units at a pre-set optical range), the greater the endotoxin concentration. This onset time range not only varies depending upon the level of endotoxin, it will vary for different lots of lysate and Control Standard Endotoxin.

It is important that the onset time falls within the correct range because it establishes that the correct dilutions have been performed. It is

Figure 1.



possible, for example, that if a test was diluted incorrectly, the correlation coefficient and the reported endotoxin values would still appear to be correct. This is because the correlation coefficient is the line of best fit between actual and expected values and the software that interprets this data and produces an estimated endotoxin concentration by extrapolating from the standard series.[13]

One way by which the range for the time taken for the optical density of the starting concentration to reach the required threshold can be calculated from the second standard deviation from the mean. Given the inherent inaccuracy of the LAL test (which is commonly accepted as ±25%), and to incorporate a level of standardization with approaches taken to other biological assays, the second standard deviation is arguably the appropriate measure. The standard deviation is a measure of how much individual elements tend to deviate from the average (mean). It is calculated as the square root of the variance (as shown in Figure 1).

The use of 2 standard deviations from the mean indicates that 95% of the values fall within the upper and lower ranges. The 5% outside of this are taken to represent atypical values.

After the completion of each LAL test, the onset time recording the highest endotoxin concentration (the start of the curve) are compared against this range and the test is only deemed to be acceptable should the measured onset time fall within this range. If a dilution error had occurred, then the onset time would have fallen outside of the expected time range.

By adopting 2 standard deviations from the mean, any test values which fall outside of the calculated range are said to be atypical values and not representative of the normal population. On this basis, the LAL test requires investigation. As a way of a further check on technician performance, the onset times for each technician's standard curve can be trended and examined by the area supervisor.

Standard Curve Correlation Coefficient

An additional check can be made on the linearity of the standard series. With this, the correlation coefficient of the standard curve should be examined for each test (where the requirement is for a correlation coefficient of 0.980 or greater). This check is required by the pharmacopoeia for each test run.

Standard curve consistency is an important feature of the LAL test. A change of only 1% in y-intercept for a linear standard curve can result in a 30% to 35% change in endotoxin determination. So, a sample with a known 10 EU/mL can read 13.5 EU/mL—not because of a change in the endotoxin content of the sample, but because of a shift in the y-intercept.[6]

Negative Controls

Negative controls should be run for each test. The negative controls are samples of the water used to construct the standard curve. The negative control samples will indicate if any contamination has occurred during the preparation of the endotoxin series. The controls are required to have an endotoxin level below that of the lowest endotoxin concentration within the standard curve (for the routine standard series, this is 0.005 EU/mL). This check is required by the pharmacopoeia.

Summary

This paper has considered some of the sources of variation which affect the LAL test. These are important for the laboratory user to understand, especially for the design of the assay and with the investigation of test anomalies. The article has also considered one key measure of variability: the coefficient of variation. This is an important check for the laboratory supervisor to include when reviewing test results.

References

1. Williams KL. *Endotoxins, Pyrogens, LAL Testing and Depyrogenation*, 2nd edition, CRC Press: Boca Raton. 2007.
2. McCullough KC and Weider-Loeven, C.: 'Variability in the LAL Test: Comparison of Three Kinetic Methods for the Testing of Pharmaceutical Products', *Journal of Parenteral Science and Technology.* 1992;44:69-72.
3. Brandberg K, et al. : Conformation of lipid A. the endotoxic center of bacterial lipopolysaccharide. *Journal of Endotoxin Research.* 1996;3:173-178.
4. Moser K. Playing Hide and Seek with Endotoxin, *LAL User Group Newsletter.* 2009;3(2),1-5.
5. Kumar H. 'Variability in the Bacterial Endotoxin Test or LAL Test', *Endosafe Times*, Charles River Laboratories, USA. 2007.
6. McCullough K. (2008): Laboratory Variability, *LAL Users' Group Newsletter.* 2008;2(3): 6-7.
7. Brosnahan K. "Understanding Correlation Coefficients and Coefficients of Variation in Photometric LAL Testing", *LAL Update.* 2006;23(2):3-5.
8. Richardson K. and Novitsky, T.J. 'Simple Statistics for the LAL User – Standard Deviation, Repeatability, Reproducibility and a Clarification of the Coefficient of Variation', *LAL Update.* 2002:20(4).
9. Lindsay GK, Roslansky PF, Novitsky TJ (1989).S ingle-step, chromogenic Limulus amebocyte lysate assay for endotoxin, *J Clin Microbiol.* 1989;27(5): 947–951.
10. Górny RL, Douwes J, Versloot P, Heederik D, Dutkiewicz J. Application of the classic Limulus Test and the Quantitatibe Kinetic Chromogenic Method for Evaluation of Endotoxin Concentration in indoor air. *Ann Agric Environ Med.* 1999; 6:45-51.
11. Tsuchiya M. "Biases in the Bacterial Endotoxin Test", *BioProcess International* Industry Year Book 2010-2011:144-145.
12. Guy D. "Endotoxins and Depyrogenation" in Hodges N. and Hanlon G. *Industrial Pharmaceutical Microbiology: Standards and Controls.* Euromed, 2003:12.1–12.15.
13. McCullough K. 'Back to Basics: Where Did My Standard Curve Come From?', *LAL User Group Newsletter.* 2009:3(2):6-8.