LAL Update®

ASSOCIATES OF CAPE COD INCORPORATED MARCH 1997 / VOL. 15, NO. 1

Letter from the President

Michael Dawson concludes his two part series on inhibition and enhancement in this issue of the LAL UPDATE. In this installment, use of spectrophotometric methods is covered. As with any method, the most important aspect is a reliable means to identify and eliminate product interference. ACC's successful introduction of the first kinetic LAL reagent, machine, and software in 1985 was the result of solving the inhibition/enhancement problem. Although the reagent, machine, and software have changed over the years, our basic method of inhibition/enhancement testing remains and has been used as a model for guidelines and regulations worldwide.

As spring approaches our thoughts are rapidly turning to horseshoe crabs. Fortunately our crab supplies are stable, because Cape Cod remains relatively pristine and undeveloped (industrially). ACC however takes an active role in the protection and conservation of the horseshoe crab. We are working to reduce the number of crabs we use (even though all are returned alive to their area of capture) and ultimately to replace the horseshoe crab. As part of our commitment to LAL research and development, we have recently begun the renovation and expansion of a new facility near our main office in Falmouth.

Sincerely,

Thomas J. Novitsky, Ph.D.

Inhibition or Enhancement Testing: Part 2

By Michael E. Dawson, Ph.D.

Introduction

The following is the second part of an article which started in the December 1996 issue of the LAL UPDATE. In the first part, the topic was introduced and regulatory issues were discussed. Attention was given to discrepancies between the various pharmacopoeia. Specifics of Inhibition/Enhancement testing of products by the gel-clot method were discussed with examples. This article addresses the specifics of the chromogenic and turbidimetric LAL methods and concludes with some general points on the topic.

Chromogenic and Turbidimetric Methods

For the chromogenic and turbidimetric methods, the product must be tested in at least duplicate, both without and with an added endotoxin spike. The concentration of the added endotoxin spike was discussed in the article on Preliminary Testing (March, 1996 issue of the LAL UPDATE). In summary, the endotoxin concentration should be 4λ for endpoint methods. For kinetic methods, it should be at or near the middle of the standard curve and should equal one of the standard concentrations. If the method of preparing the endotoxin spike in product differs from the method used to prepare the equivalent standard concentration, a positive control prepared by spiking LAL reagent water in the same way that the product was spiked is often useful.

It should be pointed out that this procedure is not consistent with the requirements of the Japanese Pharmacopoeia (JP) XIII. In essence, JP XIII states that the product must be diluted so that the endotoxin limit for the product falls in the middle of the standard curve. The product is then tested unspiked and spiked at the endotoxin limit. Spikes must be recovered within the range of 75-125% for endpoint methods and

(continued on page 2)

In This Issue...

Technical Report

Inhibition or Enhacement Testing: Part 2 1

Calendar 3

Important Announcement 4

Example – Validation Data from Pyros® for Windows®

Exported LAL-5000 data from the *Pyros* Data Summary (Table 1) shows standard curve data in rows 5–10 and samples in rows 12 and 13. Column D shows the measured endotoxin concentration (mean of replicates) in the samples (unspiked). Column E contains the endotoxin concentration in spiked samples. Spiked samples contain added spike and endogenous (contaminant) endotoxin. Endogenous endotoxin is reported (column D). Column F shows the recovered spike obtained by subtracting the value in column D from that in column E. The spike recovery, as a percentage of the nominal concentration (column C), is given in column G. The mean final concentration in column H is the measured concentration of unspiked sample (column D) multiplied by the dilution factor (column B).

Standard 1 from current file LAL lot: 12-50-642-T
Description: Endotoxin lot #: EC-5
Concentration range: 0.00100 – 0.0320 EU/ml Valid onset times: 2040 – 5560s.
Slope: -0.29015 Y intercept: 2.8748 Correlation coef.: 0.998

Endotoxin limits are not given on the Data Summary but these are shown on *Pyros* Sample Results report (Table 2) and on the *Pyros* single sample report. Both samples contain endotoxin at concentrations below the endotoxin limit.

	A	B	C	D	E	F	G	H	I
1	Sample	Sample	Std./Spk	Mean	Mean	Meas	Spike	Mean final	Coeff.
2	descrp.	dilutn.	conc.	conc.	sp.conc	sp.-un	sp.recov.	conc.	var.
3			EU/ml	EU/ml	EU/ml	EU/ml	50%	EU/ml	%
4									
5	Standard 1		0.001	0.00096					4.5
6			0.002	0.0019					4.2
7			0.004	0.0047					3.7
8			0.008	0.00765					2.7
9			0.016	0.017					9.9
10			0.032	0.03					3.1
11									
12	Sample A	2	0.004	0.00895	0.012	0.00308	77	0.0179	0.92
13	Sample B	2	0.004	0.00853	0.0125	0.00399	100	0.0171	0.32

Table 1. Data summary from *Pyros* report.
These data indicate that the test is valid and show spike recoveries well within the =/- 50% limit.

Sample descrp.	Sample dilutn.	Spike conc. EU/ml	Endtxn. conc. EU/ml	Spike recov. 50%	Mean Final EU/ml	Endtxn. Limits EU/ml	Pass/ Fail
Sample A	2	0.004	0.00895	Yes	0.0179	0.05	Pass
Sample B	2	0.004	0.00853	Yes	0.0171	0.05	Pass

Table 2. Exported LAL-5000 data from *Pyros* Sample Results.

Pyros print-outs of these and other tables show all this information plus a header with additional test details (technician ID, date, comments etc.).

Inhibition

(continued from page 1)

50-200% for kinetic methods. The JP method will not be discussed in greater detail in this article.

In the chromogenic and turbidimetric tests, there is no fixed sensitivity to the lysate. The sensitivity (λ) of a test is the least endotoxin concentration on the standard curve. Any sample containing less than λ will fall outside the standard curve and the result can only be reported as <λ. For an inhibition/enhancement test and for routine tests, λ must be less than or equal to the endotoxin limit for the product dilution being tested (the Pass/Fail Cutoff as defined in the FDA guidance—*Interim Guidance for Human and Veterinary Drug Products and Biologicals: Kinetic LAL Techniques,* FDA, 1991). If λ is not less than or equal to the limit, it cannot be stated that a product containing no detectable endotoxin (i.e., less than λ) contains less than the limit. The range of standard concentrations and the product dilution should be selected so that the endotoxin limit falls within the standard curve.

The crux of inhibition/enhancement testing is determination of the spike recovery, that is, the expression of measured endotoxin spike concentration as a percentage of the known concentration. Unfortunately there is no single, consistent rule for the required spike recovery in the regulatory documents. For endpoint methods, both chromogenic and turbidimetric, the FDA guideline (*Guideline on Validation of the Limulus Amebocyte Lysate Test as an End-Product Test for Human and Animal Parenteral Drugs, Biological Products, and Medical Devices,* FDA, 1987) states that spikes must be recovered within

+/-25% of the 4λ concentration. The more recent FDA guidance addresses kinetic methods only and states that spikes must be recovered within +/- 50% of the actual (i.e., nominal or theoretical) concentration. There is no official European Pharmacopoeial document that addresses this issue. The European Pharmacopoeial (EP, 1997) chapter, *Bacterial Endotoxin*, only addresses the gel-clot method. The EP annex (1997), *Test for Bacterial Endotoxin: Guidelines*, does address chromogenic and turbidimetric methods, but it is a guideline and is not a mandatory part of the EP. The standard curve must have a correction coefficient with an absolute value of at least 0.980.

The FDA guideline specifies at least four endotoxin concentrations; also , the difference between the OD readings for the highest and lowest endotoxin standards must be between 0.4 and 1.5 OD units. For kinetic methods, the FDA guidance specifies at least three concentrations and at least one additional per tenfold increment in the range. Standards are tested in at least duplicate. Also, the negative controls (at least duplicates) must contain less endotoxin than the lowest standard concentration. The amount of endotoxin in the sample must be less than the endotoxin limit and should not significantly interfere with the ability to discern the recovery of the endotoxin spike. The concentration of endotoxin measured in the unspiked sample is subtracted from that in the spiked sample in order to determine the measured recovery of the spike. A data summary obtained from the LAL-5000 Automatic Endotoxin Detection System is given on page 2 in the section titled "Example – Validation Data from *Pyros*® for Windows®" to illustrate some of the concepts that have been discussed in this article.

Conclusion

Successful validation is a little like painting a house. The most important work and the most effort are the preparation. In the case of validation of the LAL test for a product, that initial work is the preliminary testing. If that is performed correctly, then performance of the inhibition/enhancement test should be easy.

Finally, the question of what should be validated is often raised as the various regulatory documents specifically refer to release testing of finished product. For the quantitative methods, the requirements for routine tests are the same as for the inhibition/enhancement test. Consequently, these assays are very well controlled. A routine gel-clot test only controls for inhibition, not enhancement. However, inhibition is the more common interference and it is certainly the most significant. Perhaps, the key question is for which samples is it necessary to have inhibition/enhancement test data on file before conducting routine testing. Certainly before finished product is release tested, inhibition/enhancement tests should be completed. For samples that are routinely tested, such as certain raw materials and in-process samples, it is often appropriate to have similar data on file, though this requires judgement. Some samples, particularly those of biological origin may have widely varying levels of endotoxin and validation. The most important point is that any test performed should be controlled. This is true whether the test is for a research sample, a raw material, an in-process sample or a final release test.

CALENDAR

APRIL

April 7 - April 8

LAL Methodology and Applications Seminar and Workshop
Jakarta, Indonesia

MAY

May 4 - May 8

ASM Annual Meeting
Miami Convention Center
Booth #510
Miami Beach, FL

JUNE

June 3 - June 5

LAL Methodology and Applications Seminar and Workshop
Falmouth, MA

ASSOCIATES OF CAPE COD INCORPORATED

Creating New Horizons in Endotoxin Testing

704 Main Street ▪ Falmouth, MA 02540

For customer service call
(800) LAL–TEST or
(508) 540–3444

For technical service call
(800) 848–3248
or (508) 540–3444

Please visit us
on the internet at
www.acciusa.com

IMPORTANT ANNOUNCEMENT

Associates of Cape Cod, Inc. is upgrading to a new computer software program for business operations. The new program will allow us to serve you better by increasing our efficiency throughout our operations. Beginning in March, 1997, you will see a change in our Packing Lists and Invoices. The Packing Lists will provide more information about the products you have ordered and the Invoices will now be single sheets with a convenient tear-off section to return with your remittance.

The biggest change is new Catalog Numbers for all of our products. Each product will now have a unique number to make ordering easier and less confusing than in the past. To assist you with this change, we have provided a list below of the old and new catalog numbers. Please use the new catalog numbers when placing your orders.

Thank you for your patience during this transition. If you have any questions, please contact our Customer Service department at (800) LAL–TEST.

Old Catalog Number	New Catalog Number	Description
Pyrotell® Limulus Amebocyte Lysate (LAL) for Gel–clot Method		
100-5		Multitest 5 ml/vial (50 test) All sensitivities
	G5003	Pyrotell LAL, 0.03 EU/ml, 5 ml/vial
	G5006	Pyrotell LAL, 0.06 EU/ml, 5 ml/vial
	G5125	Pyrotell LAL, 0.125 EU/ml, 5 ml/vial
	G5250	Pyrotell LAL, 0.25 EU/ml, 5 ml/vial
100-2		Multitest 2 ml/vial (20 test) All sensitivities
	G2003	Pyrotell LAL, 0.03 EU/ml, 2 ml/vial
	G2006	Pyrotell LAL, 0.06 EU/ml, 2 ml/vial
	G2125	Pyrotell LAL, 0.125 EU/ml, 2 ml/vial
	G2250	Pyrotell LAL, 0.25 EU/ml, 2 ml/vial
100-1		Multitest 1 ml/vial (10 test) All sensitivities
	G1003	Pyrotell LAL, 0.03 EU/ml, 1 ml/vial
	G1006	Pyrotell LAL, 0.06 EU/ml, 1 ml/vial
	G1125	Pyrotell LAL, 0.125 EU/ml, 1 ml/vial
	G1250	Pyrotell LAL, 0.25 EU/ml, 1 ml/vial
100-0.2		Single test vial (STV), 0.2 ml/vial
	GS003	Pyrotell STV, 0.03 EU/ml, 0.2 ml/vial
	GS006	Pyrotell STV, 0.06 EU/ml, 0.2 ml/vial
	GS125	Pyrotell STV, 0.125 EU/ml, 0.2 ml/vial
	GS250	Pyrotell STV, 0.25 EU/ml, 0.2 ml/vial
	GS500	Pyrotell STV, 0.5 EU/ml, 0.2 ml/vial
Pyrotell®-T *Limulus* Amebocyte Lysate (LAL) for Turbidimetric Methods		
100-K5	T0051	Pyrotell-T LAL, 5 ml/vial
Pyrochrome® *Limulus* Amebocyte Lysate (LAL) Chromogenic Test Kits		
200-1	C0060	Pyrochrome LAL, 60-Test Kit
200-2	C0120	Pyrochrome LAL, 120-Test Kit
200-3	C0180	Pyrochrome LAL, 180-Test Kit
200-4D	CD060	Pyrochrome LAL, 60-Test Kit with Diazo-Coupling
Control Standard Endotoxin (CSE)		
800-1	E0005	Escherichia coli O113:H10, 0.5 ug/vl
800-3	E0125	Escherichia coli O113:H10, 125 ug/vl
800-5	E0100	Salmonella abortusequi (NP-3), 100 ng/ml, approx. 1ml/vial
800-C-1	EC010	Escherichia coli O113:H10, 10 ng/vial, for use with Pyrochrome kits
101-0.2	PC010	Positive Control for Single Test Vial, 0.2 ml LAL and CSE, CSE concentration is 0.1 ng/ml (for research use only)
Pyrosol® LAL Reconstitution Buffer		
300-1	BR051	with pH indicator (gel-clot method only), approx. 5.5 ml/vial
305-2	BC051	no pH indicator, approx. 5.5 ml/vial
355-2	BC554	no pH indicator, approx. 55 ml/vial

Old Catalog Number	New Catalog Number	Description
LAL Reagent Water (LRW)		
300-5	W0051	approx. 5.5 ml/bottle
350-5	W0504	approx. 50 ml/bottle
3100-5	W1004	approx. 100 ml/bottle
Pyrotubes® Depyrogenated Tubes		
500-1	TS050	10 x 75 mm soda lime glass for gel-clot method, 50/pkg
510-1	TB050	10 x 75 mm borosilicate glass for LAL-5000, 50/pkg
512-1	TB240	12 x 75 mm borosilicate glass (for dilutions only), 40/pkg
PyroPlate®		
280-96P	CA961	Microplate released at 0.005 EU/ml for use in turbidimetric or chromogenic methods.
PyroBlock®		
299-B1	CA371	Microplate heat block
END-X® Beads	**Endotoxin Removal Devices (for research use only)**	
END-X	B15 R0026	Set of six 2 ml microcentrifuge tubes and six receiver tubes. Removal capacity is up to 1 ug endotoxin per tube.
END-X	B52 R0501	One 50 ml tube and one receiver tube. Removal capacity is up to 20ug endotoxin per tube.
Instrumentation and Software		
LAL-5000	IN500	LAL-5000 Automatic Endotoxin Detection System - Master module with *Pyros*® for Windows® Software and Manual
LAL-5000	IN50E	Expansion module for LAL-5000 Automatic Endotoxin Detection System
970-1	S0001	*Pyros* for Windows Software
970-M	S000M	*Pyros* for Windows Manual, additional copy
970-5	S000V	LAL-5000 Automatic Endotoxin Detection System Version 1.02 Software Verification Package
Parts, Warranties, and Service		
920-GM	WTMM1	Gold Plan Extended Warranty for Master Module
920-GE	WTEM1	Gold Plan Extended Warranty for Expansion Module
920-C	CAL01	Calibration (per module)
900-1	PT011	Lamp for LAL-5000 Series I
900-1	PT021	Lamp for LAL-5000 Series II