LAL USERS GROUP NEWSLETTER

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Upcoming Event:

PMF Bacterial Endotoxin Summit

October 27-28, 2008
San Francisco

For More Information see

http://www.microbiologyforum.org/

From the Editor

Karen McCullough, MMI Associates

This newsletter is packed full of information. Glenn Gauvry, President of the Ecological Research and Development Group, who discusses the importance of conservation of Limulus and other horseshoe crab species, provides our feature article. As an adjunct, the LAL Users' Group has asked each of the four US lysate manufacturers what conservation efforts they support. Want to learn more about horseshoe crab conservation? Visit ERDG at www.horseshoecrab.org

Our experts provide insight into three questions that were asked on the PMF List. Dr. Ron Berzofsky explains conversions between EU/mL and EU/mg. Dr. Scott Sutton provides a discussion on the question of why sampling and USP monographs, and Mr. Alan Baines of Lonza discusses test verification when only one lot of product is available.

The topic of Laboratory Variability is examined in an article that provides a hint of the kinds of issues that will be discussed at the Pharmaceutical Microbiology Forum's "Lessons Learned" Bacterial Endotoxin Summit on October 27-28 in San Francisco.

One of the purposes of the Users' Group is to provide a forum for people with similar interests and concerns to network. Such is the case with the Ophthalmic Endotoxin Interest Group, a task force that has been organized under the Users' Group banner.

The American Horseshoe Crab *Limulus polyphemus:* Signs of Recovery

Glenn Gauvry, President, Ecological Research & Development Group (ERDG)

The horseshoe crab population had been dwindling in the United States. But in August, 2008 the Atlantic States Marine Fisheries Commission (ASMFC), the lead organization behind the management of the American horseshoe crab population, approved Horseshoe Crab Addendum V, Interstate Fishery Management Plan, which promotes the recovery of the horseshoe crab population, and balances the concerns surrounding the declining shorebird population, most notably a subspecies of Red knot *Calidris canutus rufa.* The Red knot is one of many shorebird species that feed upon the fat rich horseshoe crab eggs during a migratory journey that takes them from wintering grounds in Chile to breeding grounds in the Arctic. The Delaware Bay, with its abundance of horseshoe crabs, is a major refueling stop for these long distant migrants. A reduction of spawning horseshoe crabs not only impacts the medical industry, but also impacts the survival of the Red Knot species.

Horseshoe Crab Addendum V* allows a limited commercial harvest of horseshoe crabs used as bait in the conch and eel fisheries. Presently, the LAL industry remains outside of the provisions set forth in Addendum V, meaning that lysate manufacturers can still

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* http://www.asmfc.org/speciesDocuments/horseshoeCrab/fmps/hscAddendumV.pdf

Ask the Experts:

How do you convert from EU/mL to EU/mg?

Answered by: Dr. Ron Berzofsky, Wako Chemicals USA

This question is not as trivial as it may first appear. The question stems from the fact that while Endotoxin Limits in the majority of USP drug monographs are stated in EU/(units of weight), like EU/mg, the LAL test reports results in EU/ml. There actually are two answers to this question. The first is you multiply; the second is you divide.

For example: Drug A has an endotoxin limit of 5 EU/mg. Starting from an Endotoxin Limit stated in EU/mg to convert to an Endotoxin Limit in EU/ml you must know the drug concentration in mg /ml. A simple multiplication converts an Endotoxin Limit in EU/mg to EU/ml. For example, a 10 mg / ml solution of a drug whose Endotoxin Limit is 5 EU/mg = 50 EU /ml [5 EU/mg x 10 mg/ml]. The MVD (Maximum Valid Dilution) = Endotoxin Limit / lambda. If we were using a gel-clot lysate with a sensitivity of 0.125 EU/ml to test this drug, the MVD = 50 EU/ml divided by 0.125 EU/ml = 1/400

If you're starting with a dry powder bulk for Drug A and will prepare the test solution in the lab, you need to calculate the MVC (minimum valid concentration). The formula for MVC is lambda / Endotoxin Limit. Using a gel-clot lysate with a sensitivity of 0.125 EU/ml to test Drug A with an Endotoxin Limit of 5 EU/mg, our MVC would be 0.025 mg/ml [0.125 EU/ml divided by 5 EU/mg], meaning we could not test a solution less concentrated than 0.025 mg /ml. **NOTE**: A 1/400 dilution for Drug A (our MVD for a 10 mg/ml solution) would generate a 0.025 mg/ml solution –our MVC.

Once we performed a LAL test on a drug solution and obtain an EU /ml result, to determine EU /mg you divide by the drug concentration in the test sample. For example, if we used a kinetic LAL assay to test a 0.1 mg/ml solution of Drug A and obtained a 0.05 EU/ml result, the corresponding EU/mg = 0.5 EU/mg [0.05 EU/ml divided by 0.1 mg/ml].

"Validation" or "verification" of BET to demonstrate the suitability with our product requires three different batches of product for inhibition/enhancement. If we don't have three different batches, do you think that it is acceptable to perform three independent assays on one batch if only one batch is available?

Answered by: Mr. Alan Baines, Lonza

The pharmacopeial test for the Bacterial Endotoxin Test (BET) describes clearly the procedures for validation of a product, with a view to establishing a suitable dilution, with or without additional treatment when necessary, within the Maximum Valid Dilution (MVD) that can be used on a routine basis to perform a final product release test for endotoxin. The monograph indicates that three batches of product are required to confirm that the laboratory can use the chosen dilution with reasonable confidence for final product release.

The standard procedure is to carry out an inhibition/enhancement assay usually using a series of dilutions starting at the MVD and working backwards. This establishes an 'interference profile' for the product and from this profile, a suitable dilution can be chosen that demonstrates minimal interference with the LAL test. Once a suitable dilution has been determined, three batches of product are tested at the chosen dilution, using a Positive Product Control (PPC). If the recovery of the PPCs are within the permitted limits, demonstrating that there is no interference from any of the three batches and that the test results are valid, then the laboratory can proceed, after suitable documentation, to test and release product using the selected dilution. However, a Positive Product Control must be included with each test. The reason for the requirement for three separate batches of product in the second stage of the validation exercise is to establish that there is insufficient variation in the formulation of the product, on a batch-to-batch basis, to invalidate the original data.

However, there are a number of instances where there may not be three batches of product available to carry out the three-batch validation of the chosen dilution. This typically occurs in circumstances such as the production of early batches for Clinical Trial or Clinical

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Ask the Experts

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Trial Exemption purposes or perhaps if the product is produced so infrequently, that there are practical problems in assembling three batches of product for validation testing. How then should a manufacturer proceed in this case?

As there is insufficient data available to confirm consistency batch-to-batch with the product under consideration, it is necessary to carry out a full inhibition/enhancement assay on the first and any subsequent batches of product until there is data from three separate batches. The use of an inhibition/enhancement assay will establish one or more dilutions where there is no significant interference and the endotoxin content of the sample can be reliably reported. Users should be careful to note that where there are multiple results available from 2 or more dilutions that meet the PPC recovery requirements, that there are no contradictory results. In this case further work is necessary to determine the cause of the contradictory data.

The use of an inhibition/enhancement assay with normal assay controls, will allow the testing and release of product in such cases, where the full 3-batch validation has not yet been carried out.

Looking in the European Pharmacopeia 2.6.14, in USP <85> and JP XIV 6. Bacterial Endotoxins test, I could find no guidance about the number of samples to be tested. But FDA's "Guideline on Validation of the Limulus Amebocyte Lysate Test ..." (1987) says to test "a minimum of three units, representing the beginning, middle, and end …from a lot. These units can be run individually or pooled". Why there is nothing about sampling in the different pharmacopeia ?

Does everybody follow the 1987 Guideline ? Can an Investigator question testing only one sample from a batch?

Answered by: Dr. Scott Sutton, Vectech Consulting and Vice-chair, Expert Committee on Microbiology and Sterility Assurance

Question #1: Sampling is a difficult question for the compendia, particularly when discussing the microbiology assays. The problem with relying on a finished product test for determination of a quality attribute (sterility, LAL, etc) is that we really cannot sample enough units in a batch to demonstrate, statistically, that the attribute requirement is met (a recent discussion of the Sterility Test in that regard can be found in Moldenhauer and Sutton. 2004. Towards an Improved Sterility Test. *PDA J Pharm Sci Tech*. 58(6):284-286).

The *General Notices* section in USP contains a great deal of useful information. In there we find that chapters numbered less that 1000 exist to support the product label claim that a compound or pharmaceutical product meets USP monograph requirements. Thus, the Bacterial Endotoxins Test (USP <85>) is a referee test that exists only to support those monographs that include a requirement to pass the Bacterial Endotoxins Test. Similarly, the Sterility Test exists to support those monographs that require compliance with the sterility test. Results of these assays attest to the fact that the sample tested meets the requirements.

If USP tests are not intended for a finished product release, why provide sampling instructions for the Sterility Test or for the Microbial Limits Tests and not the Bacterial Endotoxins Test? The deeply unsatisfying, but accurate answer is that the compendial tests are not consistent in this regard. This inconsistency may be traced to their development at different times by different groups of people. It might also be traced to their role as referee tests for different product characteristics and that testing for these may require consideration of the sampling plan due to the nature of the characteristic being tested. That is to say, sterility failures would be due to viable organisms at low numbers, and sampling error will play a big role in our ability to "see" these events – therefore sample size is a major component of the test. The BET is looking for a compound in higher numbers (relatively speaking) and so sampling error (as a component of the test) is less of a concern.

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Question #2: This pair of questions is much easier to answer. The 1987 Guideline is the only guidance that has been published on this topic by FDA and is a joint publication of CDER, CBER, CVM and CDRH. The three vials, one each from the beginning, middle and end is a minimal expected sampling, and most companies follow the suggestion for testing drug product. However, since this is only a Guidance, people can discuss variations from acceptance practice with FDA. If you wish to do less testing than this guideline recommends I would strongly urge that you have a well-documented and scientifically sound, written justification in place and that your legal department has signed off on the practice. There might be valid reasons to do less testing than three units (batch size of 1 unit, for example). Of course, exceeding the guidance would be a far less risky practice.

Do you have a question for our experts, or would you like to contribute a paper to the Users' Group Newsletter?

Write to us at http://www.microbiologyforum.org/laluser.htm

The American Horseshoe Crab *Limulus polyphemus*, Signs of Recovery

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collect crabs for bleeding and return them to their natural habitat once the process is complete.

As with all ASMFC plans, individual states can adopt more conservative management practices. In the case of New Jersey, a full moratorium on the harvest of horseshoe crabs has been in place since 2006. However, a balanced conservation strategy will require more than harvest management.

Through the efforts of individual states and ASMFC, the horseshoe crab population in the United States shows signs of recovery, the same can not be said for the worlds three other species, which are in various states of decline. Many inhabit places where human considerations often over shadow environmental concerns, and regulations, if they exist, lack enforcement. Some, particularly in Southeast Asia and India are coming under increased pressure as opportunistic harvesters discover their catch is worth more to the biomedical industry than for human consumption or bait. This trend will expand proportionately to demand. In the recently published book "Sustaining Life, How Human Health Depends on Biodiversity" editors Eric Chivian and Aaron Bernstein, include the horseshoe crab as one of many species of concern.

The future survival of the world's four horseshoe crab species will ultimately depend upon the preservation of spawning habitat; a challenging prospect in light of the ever-increasing human density along the same beaches horseshoe crabs rely on for propagation. With few exceptions, horseshoe crabs do not spawn within protected habitats. They come ashore where humans live, play, and work. Sometimes they are accepted, but more often they are exploited or even reviled; numerous horseshoe crabs die from stranding on beaches during the spawning season each year, and their carcasses create an odorous and untidy shoreline, often in areas where the value of luxury homes and vacation destinations depend on a "pristine" environment. It is here, within these coastal communities, that horseshoe crab conservation can be most effective. Driven by science as well as economic considerations and strengthened by compassion for both the natural resource and the community, a conservation framework can be established.

What role, if any, does the LAL, biomedical and pharmaceutical industries have in the conservation of a species it depends upon for advancements in human health and safety? Should it become engaged in the formulation and implementation of regulatory processes designed around sustainability? Or is it better to support those who have an active hand in the conservation process and have demonstrated an understanding of the industries need for access to this remarkable mariner? *(Editor's Note: The good news is that US lysate manufacturers are actively involved in many ways. See accompanying feature, "Lysate Manufacturers Contribute to Conservation")*

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The American Horseshoe Crab *Limulus polyphemus*, Signs of Recovery

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Although it is easy for people to take sides in any conservation issue, it is far more effective and productive if all sides can work together to find ways to solve a problem. It requires more time, effort, and innovation, but in the end, it helps to build a cooperative community of problem-solvers who will readily accept the solutions they themselves have had a hand in creating.

Building on the belief that conservation is an informed individual's responsibility, which establishes the foundation for community-based conservation, the Ecological Research & Development Group (ERDG), a nonprofit organization dedicated to the conservation of the world's four horseshoe crab species, created *The Horseshoe Crab Conservation Network*™. The network is a multifaceted, cross-cultural suite of initiatives designed to inform and engage the broadest coalition of individuals, communities, organizations, and scientists in the conservation of the world's four horseshoe crab species. More can be learned about this organization at www.horseshoecrab.org.

Lysate Manufacturers Contribute to Conservation

We asked US lysate manufacturers to tell us how their organizations are involved in horseshoe crab conservation efforts. Their answers are provided below:

From Associates of Cape Cod: "Associates of Cape Cod, Inc. (ACC) serves on the local Horseshoe Crab Conservation Committee and has been instrumental in influencing State regulation to increase protection of horseshoe crabs. ACC cooperates with the State Division of Marine Fisheries in their work to regulate the horseshoe crab fishery and is represented on the Advisory Panel to the Horseshoe Crab Management Board of the Atlantic States Marine Fisheries Commission. ACC is regularly involved in education programs, both independently and with other organizations, including the Massachusetts Audubon Society and the Green Eggs and Sand Program run by the Maryland Department of Natural Resources."

From Charles River Endosafe: "Charles River is in a unique position regarding the horseshoe crab population in South Carolina (the location of Charles River's crab bleeding facility). In 1992, Dr. James Cooper (the company founder) and his wife Francis initiated a dialogue with the Atlantic States Marine Fishery Commission (ASMFC) and the South Carolina Department of Natural Resources. That dialog resulted in state legislation that protects the indigenous horseshoe crab population. In South Carolina, horseshoe crabs can only be used for biomedical applications (LAL production) and marine biological research. In South Carolina, the horseshoe crabs cannot be used as bait for the eel and whelk industries. As a result, the horseshoe crabs are more protected in South Carolina than anywhere else in world. This proactive legislation and the controlled (and monitored) bleeding and horseshoe crab handling procedures have created a significantly increasing population of horseshoe crab in South Carolina."

From Lonza: "At Lonza, we are aware of the importance of the horseshoe crab for its contribution to the safety of drugs and medical devices but also its role in the circle of life. Lonza does not sell the horseshoe crabs that we use for biomedical purposes after bleeding, they are returned alive to the same waters from which they were taken. We sit on the Atlantic States Marine Fisheries Commission (ASMFC) Technical Committee for the horseshoe crab and its joint team with the Shorebirds Commission. Annually we donate funds to support educational programs and help coastal communities save the horseshoe crab breeding grounds. In addition, we have developed an alternative endotoxin detection assay that does not rely upon horseshoe crab blood. The PyroGene® assay uses a recombinant form of the horseshoe crab Factor C to detect endotoxin. With the PyroGene® assay, there is less dependence on the animal and less animal utilization. At Lonza, we strive to help protect the future generations of the horseshoe crab."

From Wako Chemicals, USA: "Wako USA is very much concerned about maintaining the viability of the horseshoe crab population. Wako USA commissions an independent fisherman to obtains horseshoe crabs daily (weather permitting) in ocean waters about 1 mile off the shore of Assateague Virginia. The crabs are collected using nets to minimize injury. After bleeding the crabs are returned the next day back to the same waters where they were collected. Wako USA participates in a horseshoe crab tagging and monitoring program coordinated by the Virginia Institute of Marine Science."

Bacterial Endotoxin Summit Preview: Laboratory Variability

Karen McCullough, MMI Associates

"There is no SOP for tracking laboratory errors." So goes a 483 observation that applies to a BET laboratory. Errors are, in part, a function of variability. The section, "Initial Qualification of the Laboratory" in the 1987 FDA Guideline states, "Manufacturers should assess the variability of the testing laboratory before any official tests are performed." What does that mean?

Analysts
Reading on a little further in the Guideline, the text qualifies this statement by instructing that each analyst, using a single lot of LAL and a single lot of endotoxin should perform the test for confirmation of labeled LAL reagent sensitivity or of performance criteria. So, the first thing to control in terms of variability is the analyst. Analysts, though impeccably trained, are perhaps the most difficult of all variables to manage. Human nature is such that each analyst has his/her own subtle technique for laboratory tasks such as making dilutions, pipetting, and weighing raw materials. Good aseptic technique is a must. The Pharmacopeia do not require analyst qualification, but GMP, the FDA Guideline and Common Sense do require that all laboratory analysts be trained and demonstrate competency prior to performing tests. The USP sections, "Test for Confirmation of Labeled LAL Reagent Sensitivity" and "Verification of Criteria for the Standard Curve" describe methods that can be used for analyst qualification for gel clot and quantitative tests, respectively.

What else can contribute to variability?

Reagents
Remember, any solution that you use in the performance of the BET can contribute to variability. The USP sections for confirmation of label claim and verification criteria for the standard curve will demonstrate that the lysate, endotoxin and LRW are all working together appropriately. But what about buffers that you might add to your test to stabilize pH? What about divalent cations that you might add? Making certain that all ancillary solutions are free of detectable endotoxin will help reduce variability.

Consumables
Validation of depyrogenation of heat-stable accessories used in BET is a given. But recognizing that plastics used in the performance of the BET (e.g. microtiter plates, plastic dilution tubes) are not made specifically for endotoxin testing, the USP section, "Apparatus and Glassware" requires that all plastics be screened for test interference. Ever encountered a "hot well"? The high temperatures involved in melting/molding plastic should eliminate endotoxin, but handling and the environment after the molded plastics are released could result in inadvertent and somewhat random endotoxin contamination. On the flip side, some plastic formulations can leach inhibitors that interfere with the test. For a good discussion of interferences observed in plastics, see the article, "Problems with Plastics" that was published in 1988 by Associates of Cape Cod in their newsletter.*

Standards
Do you make a set of dilutions and save the dilutions for a period of time that exceeds one testing day? Have the storage conditions (temperature, vessel composition, dilution range, volume of the dilution) been validated? If you store standard dilutions and do photometric testing, keep a close eye on shifts in onset times that could signal shifts in the potency of stored dilutions.

Standard Curves
Qualification of reagents, analysts, and verifications that confirm lack of interference by test articles all require that label claim, lambda, be confirmed. Lambda is the sensitivity of the test, as identified by the lysate manufacturer, confirmed by FDA, and re-confirmed by the testing lab. If dilutions are off, if articles interfere or if the heat block gives out, lambda will not be confirmed, sending an instant message regarding variability to the lab.

The requirements are different for photometric testing Qualification of reagents and analysts and verification to confirm lack of interference by test articles by gel clot don't require the lab to match anything. All that is required is that the standard curve is linear. Dilution errors can result in linear standard curves. Errors in reconstituting reagents can result in linear standard curves. Variations in temperature can result in linear standard curves. But, linearity is not the only important standard curve attribute. A change of only 1% in y-intercept for a linear standard curve can result in a 30-35% 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. The trick to controlling variability in a photometric 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 your test result.

Sample Handling
Composition of vessels and storage conditions can affect endotoxin (see the ACC article referenced

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* http://www.acciusa.com/pdfs/newsletter/LAL_Vol.6No.3.pdf

Laboratory Variability

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above). What kinds of containers are used to take or store your samples? Are the samples sterile or are they intermediates with some level of microbial load? Are analysts trained to take samples aseptically? Do the samples need to be held under refrigeration? How long can the samples be held before testing? For a discussion on holding samples, see Guilfoyle, et al., 1989.

Instrumentation

If you're doing photometric tests and if you have multiple instruments, do you know anything about instrument-to-instrument variability? Have you compared data from the same sample from all instruments? Are they truly comparable or interchangeable?

All of this comes down to trends and patterns that are ,by definition, indicators of variability. Do you see endotoxin stability issues with a particular type of vessel? Do you notice that one analyst is the one who always reports out "hot wells"? Do you notice that a good number of invalid test results come from one instrument? Do you notice that the same analyst takes samples of all of the high, out of trend water results? Do you notice that Friday afternoon has more than its share of invalid tests?

Lessons Learned... Identify all high risk variables in the laboratory and sample handling. Understanding variability, tracking/trending laboratory errors and invalids and increasing control over many aspects of your BET assay will reduce inconsistency and increase accuracy. Want to continue the discussion? Go to http://www.microbiologyforum.org/laluser.htm and learn more about the Bacterial Endotoxin Summit.

Literature cited:

Guilfoyle, Dennis, James F. Yager, Sebastian L. Carrito. 1989. The effect of refrigeration and missing on detection of endotoxin in parenteral drugs using the Limulus Amebocyte Lysate (LAL) test. J Parent Sci Tech 43(4): 183-187

GMP Watch

21 CFR 210 and 211 Amendments to the Current Good Manufacturing Practice Regulations for Finished Pharmaceuticals, Final Rule, will become effective December 8, 2008. The following endotoxin-related item is from the Federal Register:

"Revised 211.94(c) requires validation of depyrogenation processes for drug product containers and closures, consistent with longstanding industry practice and agency interpretation of this regulation."

For more information on longstanding industry practice, see the following:
USP <1211>, "Sterilization and Sterility Assurance of Compendial Articles"
LAL Users' Group. 1989. Preparation and Use of Endotoxin Indicators for Depyrogenation Process Studies. J Parent Sci Tech 43(3).

Who are these happy people? Here are the answers to the test of your knowledge of LAL history. Starting from the top left: Dr. Ron Berzofsky, known for introducing chromogenic testing and the Power Curve to the BET now at Wako Chemicals USA; Mr. Terry Munson, author of the 1987 Guideline, former Chief, Sterile Drug Branch, Division of Manufacturing and Product Office of Compliance at the Center for Drug Evaluation and Research (CDER), now at Parexel Consulting; Karen McCullough, first to publish on the use of LAL with small volume parenterals, co-founder of the LAL Users' group and still on the Steering Committee; Dr. James F. Cooper, first to publish on the use of the LAL test with radiopharmaceuticals and founder of Endosafe, now an independent consultant.

About The LAL Users' Group...

The LAL Users' Group, and the Users' Group Newsletter provide a forum for discussion of issues and advances in the arena of the Bacterial Endotoxin Test. The information contained in this newsletter includes the professional opinions of individuals and does not represent the policies or operations of any corporation or government agency to which they may be associated. *LAL Usrs' Group Newsletter* is intended to serve as an open forum and solely for informational purposes, developed from sources believed to be reliable. Articles or opinions are for information only to stimulate discussion and are not necessarily the views of the PMF board or regulatory agencies. The *LAL Users' Group Newsletter* cannot make any representations as to the accuracy or completeness of the information presented and the publisher cannot be held liable for errors.