

ENDOSAFETIMES

In Vitro Products and Services Newsletter


CHARLES RIVER
LABORATORIES

Volume 13, No. 1, April 2007



PERSPECTIVE

James F. Cooper, Ph.D., consultant

This issue of *Endosafe Times* focuses on the early history of pyrogen testing and more information about the latest advancement in endotoxin testing, the Endosafe®-PTS™. We are privileged to have Marlys Weary briefly recount the fascinating story of the complicating role of pyrogens during the development of intravenous (IV) therapy. Although the notion of delivering medications by the IV route was pursued for almost four centuries, critical observations were needed to solve the pyrogen puzzle and make injectables a safe reality. The fever reactions and cardiovascular shock associated with IV medications were addressed by recognizing the bacterial source of pyrogen, developing ways to eliminate pyrogens from water, and using the rabbit pyrogen assay to screen for unsafe solutions. It is hard

to believe that IV therapy, as we know it today, had such recent origins. The advent of the LAL test was the last critical piece of the puzzle because it provided a simple, sensitive, and specific way to monitor parenteral drug production for endotoxin from start to finish.

The evolution of endotoxin testing

As previously announced, the precalibrated LAL reagent in Endosafe®-PTS™ was licensed by the FDA last year for all LAL applications. The crowning achievement of this innovation is that PTS™ enables endotoxin testing without the variation that accompanies other BET methods. It allows reliable

endotoxin testing in the absence of skilled BET analysts. Where BET procedures already exist in LAL test labs, validation of PTS™ methods as an additional method is simple. No product screen or characterization is required because this data was previously collected. One simply proceeds to product-test validation using an established test dilution or concentration. Valid recovery of the positive control for three batches constitutes method validation for specific products. Of course, BET product characterization is indicated for defining the suitability of PTS™ for a new chemical entity. The *Laboratory Notebook* section in this issue summarizes the steps for validating Endosafe®-PTS™ as an alternative or new BET application.

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A SHORT HISTORY OF PYROGENS AND PYROGEN TESTING

Marlys Weary, MERIT Consulting Services

Pyrogens are substances that produce fever in man and many other mammals when sufficient numbers of these substances are introduced into the circulatory system. Therefore, the history of intravenous therapy and the history of pyrogen research are closely related.¹

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LAL POINTERS pH and the PTS™

Endosafe® reagents contain substantial buffer capacity so that neutralization of drug products prior to LAL testing is seldom needed. Nevertheless, pH measurement of an aliquot of a test sample, usually diluted, is a regulatory and compendial expectation when validating an LAL reagent for a product. The BET recommends pH measurement of the test sample/LAL mixture to confirm neutral reaction conditions, where pH of the mixture falls in the range of 6-to-8. Of course, testing such a mixture is not physically possible because the PTS™ has dried reagents. Since inadequate recovery of the positive control (PPC) is a sensitive indicator of non-neutral test conditions, pH measurement is only indicated if recovery is low. When sample pH is outside the PTS™ recommended range of 6.7 to 7.3 and spike recovery is low, pH adjustment of the sample is justified. Arbitrary adjustment of pH for an unbuffered solution is unwise.

A SHORT HISTORY OF PYROGENS AND PYROGEN TESTING

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The history of intravenous therapy usually dates to the early 1600s, beginning with Sir William Harvey's research studies on the circulation of blood. The possibility of using the vascular system as a carrier for therapeutic agents has been attributed to Sir Christopher Wren, who, in 1656, used a goose quill attached to a pig's bladder to intravenously administer wine, ale, and morphine to a dog.² Shortly thereafter, *Clymatica Nova* was printed in Holland in 1670, illustrating the flow of venous blood and a technique for infusing solutions into the arm and leg of humans.³ Many documented studies of intravenous therapy can be found in medical research studies that were performed during the 18th and early 19th centuries. However, this therapy was inevitably accompanied by febrile responses in the patients. In fact, intravenous therapy was often administered to patients to deliberately cause fevers, since it was thought that fevers could have beneficial effects in activating the body's defense against disease.

Although physicians recognized that intravenous therapy produced febrile, or pyrogenic reactions in their patients, they had no understanding of the mechanism by which those fevers were produced. It was known that septic fever, or wound fever frequently occurred when

LABORATORY NOTEBOOK Endosafe®-PTS™ Product Validation

Method validation for a BET (Bacterial Endotoxins Test, USP Chapter <85> or equivalent Pharmacopeial chapter) is performed to document that a specific LAL reagent or test system will detect endotoxin in a specific drug formula or device extract without interference from the sample matrix. The Endosafe®-PTS™ cartridge was licensed by the FDA in July 2006 as an LAL system for detection of bacterial endotoxin. Application of this new reagent cartridge for end-product release requires validation in accordance with the interference test for photometric methods under *Preparatory Testing for the Photometric Techniques* section of the BET chapter.

Validation of Endosafe®-PTS™ as an additional method is quite simple. We outline two approaches; one for samples already validated with an existing method and another for new chemical entities. Satisfactory recovery of the PPC (Positive Product Control) for three batches of product or extract is the basis for BET validation using the PTS™ method. Successful recovery of the PPC also indicates that buffers in the LAL reagent satisfactorily resolve pH issues. It is useful to record the pH of test sample (usually diluted) with a similar Endosafe® LAL reagent.

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tissue from open wounds or surgical sites decomposed, so physicians speculated that pyrogens might be formed in those tissues from processes such as fermentation or putrefaction. Then when Louis Pasteur (1822-1895) discovered that microorganisms were the infectious agents that caused fermentation and putrefaction, there was additional speculation that pyrogens were either associated with bacteria or were inherently a part of the bacteria themselves.

In 1889, Roussy successfully separated a crude fever-producing substance from Gram-negative bacteria.⁴ Five years later, Centanni successfully extracted pyrogenic substances from a large variety of bacteria, including *E. coli* and typhoid.⁵ Centanni also showed that these bacterial pyrogens were not proteins, and that they were heat stable. Investigations published by Hort and Penfold in 1912, provided additional important observations about the nature of injection fevers.^{6, 7, 8} These researchers used rabbits to standardize an assay for fever and then assayed various kinds of bacteria for their ability to cause fever. By utilizing the staining procedure described by the Danish physician, Hans Christian Gram in 1884, they were able to demonstrate that the pyrogenic bacteria were primarily Gram-negative, while the non-pyrogenic bacteria were Gram-positive. They also showed that dead Gram-negative bacteria were just as pyrogenic as their live counterparts, and they concluded that the cause of all injection fevers was a filterable, heat-stable bacterial substance.

Unfortunately, the work of Hort and Penfold did not receive wide circulation, and it was the classic investigations of Florence Seibert in 1923-1925 that established conclusively that injection fevers were caused by heat-stable, filterable components of Gram-negative bacteria, or what we know today as



bacterial endotoxin.^{9, 10, 11} Seibert was a hospital pharmacist at the University of Chicago who successfully developed a process to consistently produce intravenous solutions that could pass rabbit assays for injection fevers.^{12, 13} Her methods for producing non-pyrogenic intravenous solutions enabled hospitals to consistently produce these solutions for safe use in patients. Her manufacturing methods also launched the commercial large volume parenteral (LVP) solutions industry during the decade just prior to the outbreak of World War II.

The heavy demand for LVP therapy, particularly during World War II, and the need to ensure that commercial parenteral solutions were free from pyrogen contamination caused the United States Pharmacopeia (USP) to undertake the development of a compendial test for pyrogens. In 1941, The Committee of Revision of the USP authorized Subcommittee 3 on Biological Assays to begin the first USP collaborative study of pyrogens. Using *Pseudomonas aeruginosa* filtrates prepared by the Division of Bacteriology of the FDA, the collaborative study was undertaken by the FDA, the NIH, and 14 pharmaceutical

manufacturers.^{14, 15} The study utilized a rabbit assay similar to that used earlier by Hort and Penfold and Seibert and her co-workers. Large numbers of rabbits were challenged intravenously with both pyrogenic material and non-pyrogenic physiologic saline solution. The results of the study, which was published in 1943, led to the inclusion of the first compendial pyrogen test in the 12th edition of the *United States Pharmacopeia* (USP) in 1942. Although refinements have been added from time to time, the pyrogen assay described in the current USP edition and other international compendia, follows the same basic format as the original USP rabbit

Intravenous therapy led to pyrogen research

pyrogen test. Basically, the rabbit pyrogen test involves measuring the rectal temperature of rabbits, both before and after the intravenous injection of a test solution. If the animals exhibit febrile responses that exceed established limits, the test solution is judged to be pyrogenic.

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We now know that fever is only one of many physiologic responses that result from the intravenous infusion of pyrogens into the human circulation. However, during World War II, and for many years thereafter, fever was the easiest side-effect of bacterial endotoxin to detect in a validated test system. The rabbit pyrogen test continues to have one major advantage over other pyrogen test systems in that it can best replicate and demonstrate the production of fever in humans. However, the rabbit test is not specific for one class of pyrogens. It detects all kinds of injectable substances that can cause fever, not just bacterial endotoxins or endogenous cytokine pyrogens. The test also has many disadvantages. It is a time-consuming, elaborate, expensive procedure that requires a large capital investment in animal housing, trained animals, and skilled animal handlers and technicians. It can not be used to test certain toxic drugs or drugs that depress fever. Also, tolerance to certain classes of pyrogens can develop in rabbits, and like all animal tests, the rabbit pyrogen test suffers from the wide variety of responses imposed on it by biological variation.



For many years, the pharmaceutical industry searched for an alternative test method to the rabbit pyrogen test. The best answer to date has been the Limulus amoebocyte lysate test, commonly referred to as the LAL test. For information about the history and utilization of the LAL test for bacterial endotoxin pyrogens, check out the next issue of this newsletter for the continuing saga of the history of pyrogen testing.

The best alternative to the
rabbit pyrogen test is the
LAL assay.

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LABORATORY NOTEBOOK

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The steps for validating an existing test method for PTS™ include:

1. Confirm the standard curve range of a PTS™ cartridge to allow testing a product within the endotoxin limit.
2. Prepare three (3) batches of a sample at the dilution or concentration currently in use as a non-interfering preparation.
3. Test these sample preparations on the PTS™ system, one batch per cartridge.
4. Document that PPC recovery indicates that the pH of the product and test sample mixture was within a range of 6.7 to 7.3.

Once the results are recorded and the acceptance criteria are met, the validation is complete. The acceptance criteria include (a) spike recovery of the positive product control within the FDA and compendial acceptance range of 50-200% and (b) the Coefficients of Variation (CV) for reaction times for both the sample and the Positive Product Control (PPC) replicates are less than 25%.

The steps for validating new chemical entities or non-validated LAL methods include an initial screening phase:

1. Determine the Endotoxin Limit (EL).
2. Calculate the Maximum Valid Dilution (MVD) or Minimum Valid Test Concentration (MVC) based on the Endotoxin Limit and test sensitivity.

3. Test sample at several different dilutions or concentrations allowing for a 2- to 4-fold safety factor from the Endotoxin Limit, if possible. (Note: Charles River's R+D Inhibition/Enhancement four (4) spike cartridge can be used for the initial step. Follow screening by using a licensed cartridge to verify sample preparation.)

With the results from this screening, a non-interfering sample preparation for use during the validation can be determined. To complete the validation, follow the steps detailed above for an existing test method.

Product validation of the PTS™ entails the same steps that are followed for other LAL kinetic/photometric methods. Upon completion of the validation, endotoxin testing is streamlined considerably as analysts no longer have to prepare endotoxin standards and positive product controls. The lot-specific calibration code associated with each particular batch of cartridges provides information for the archived standard curve and spike concentration for the positive product control.

See tables 1 and 2 for an example of a product validation including an interference screen.

Table 1
Interference Screen for a Pharmaceutical

Dilution with LRW	Limit of Detection (EU/mL)	Spike Recovery
1:4	<0.2	22%
1:8	<0.4	7%
1:16	<0.8	16%
1:32	<1.6	44%
1:64	<3.2	65%
1:128	<6.4	68%
1:256	<12.8	76%
1:512	<25.6	84%
1:1024	<51.2	80%

Table 2
Three lot validation of a product

Batch	Dilution	LOD (EU/mL)	Spike Recovery
A	1:256	<12.8	83%
B	1:256	<12.8	88%
C	1:256	<12.8	87%

The product had an endotoxin limit of 100 EU/mL and there was no experience testing it with LAL. There was a pH issue that was resolved by dilution with LRW and the buffers present in the dried reagents in the PTS™ cartridge. A series of dilutions were tested in the PTS™ system and revealed a resolution of the LAL-test inhibition at a dilution of about 1:256, as indicated by valid spike recovery (Table 1). The product was then validated at a dilution of 1:256 using a lot of PTS™ cartridges with a standard range of 5-to-0.05 EU/mL. With a lambda of 0.05 EU/mL, the Limit of Detection was <12.8 EU/mL, well within the endotoxin limit of 100 EU/mL. Validation was conducted by testing three batches of the new drug and achieving valid recovery of the positive controls (Table 2).

Meet our North American Field Technical Specialists

Our Field Technical Specialists work closely with our Account Managers to provide exceptional, face to face support to our customers. We'd like to introduce you to two from the team who travel across the US and Canada to customers' facilities.

Kevin Klim has been with Charles River for over seven years providing technical assistance and performing reader installations and requalifications. Kevin is skilled at performing on-site product validations and often provides training to technicians new to endotoxin testing. With a BS in Biology/Chemistry and seven years of experience performing endotoxin testing at JRH Biosciences, Kevin has a strong technical background and rich practical experience. Kevin can be reached at Kevin.klim@crl.com.

Brad Parish has been with Charles River for six years and assists and trains both new and experienced customers who are performing endotoxin testing. He excels at solving issues as he worked in Endosafe Technical Service for four years and has broad experience with all issues related to LAL. Recently, he has focused his efforts on the PTS™ and provides hands-on support for this system. Prior to his work in the Technical Support Department, he was a formulation chemist. Brad has a BS in Chemistry. To reach Brad, please email brad.parish@crl.com.

In future issues, we will continue to introduce you to the many faces providing technical support for our customers around the world.



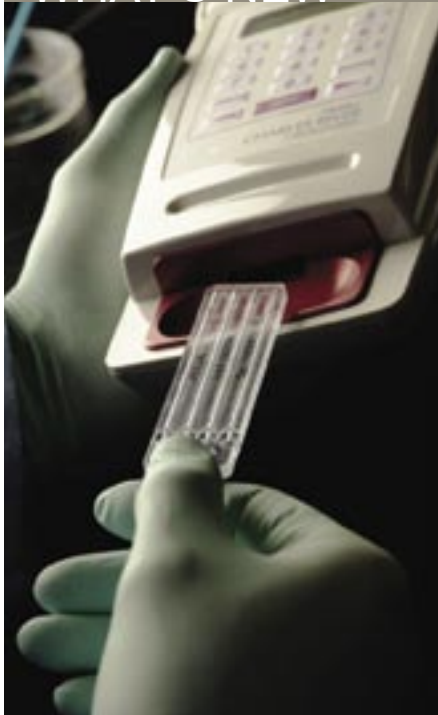
Kevin Klim, Sr. Technical Specialist



Brad Parish, Technical Specialist

Please visit us on our website www.criver.com/pts to see our latest PTS™ videos: “PTS™ - the evolution of endotoxin testing”, the PTS™ flash video, and a video of Dr. Norm Wainwright, Director of R&D for Charles River Laboratories, using the PTS™ in zero gravity.

WHAT'S NEW



PTS™ Gram ID Now Available from bioMérieux

CRL has granted bioMérieux exclusive rights to sell the PTS™ Gram ID system in the US and Canada. Charles River Technical Staff will continue to service customers and provide support. To place an order, please call 1.800.634.7656. For technical support, please call 1.800.762.7016.

Endosafe®-PTS™ Inhibition/Enhancement LAL cartridges now available

The PTS™ Inhibition / Enhancement cartridges are a helpful and cost-effective tool for screening samples to find a non-interfering dilution factor. Each cartridge contains four (4) channels loaded with positive product control allowing four sample dilutions to be tested simultaneously. The cartridges are packaged in groups of ten. When ordering, please reference item code PTS220.



Please visit at us at the following seminars and meetings:

Date	Meeting/Workshop	Location
June 2-6	SNM Annual Meeting	Washington, DC
June 5-8	LAL Training Workshop	Lyon, France
June 12	LAL Training Seminar	San Diego, CA
August 21-24	LAL Training Workshop	Charleston, SC
September 18-19	LAL Training Workshop	Potsdam, Germany
September 18-21	LAL Training Workshop	Lyon, France
September 24-26	LAL Training Workshop	Stratford-upon-Avon, United Kingdom
October 2-5	LAL Training Workshop	Lyon, France
October 9-11	Biotechnica	Hannover, Germany
December 4-7	LAL Training Workshop	Lyon, France

For more information, please visit our web site, www.criver.com, call 1.877.CRIVER1, or email us at endo-comments@crl.com.

The USP proposed changes to the BET have appeared in the Pharmacopeial Forum. During this time period for comments, Charles River encourages LAL users to review the proposed changes and provide comments to ensure that the best interests of your company and the technology are represented.

PTS™ IN SPACE

NASA recently released a favorable report on their testing using the Endosafe®-PTS™ system aboard the International Space Station (ISS). Astronauts are presently using a modified PTS™ system on the ISS. The PTS™ may be used in future space voyages to monitor electronics and structural materials for the presence of bacteria or fungi which can corrode or damage some components. In the future, the PTS™ may be a useful tool for checking the health of astronauts on long space voyages.



photo courtesy of NASA

Introducing the new multi-cartridge system, the Endosafe®-MCS™

Charles River's latest innovation, the Endosafe®-MCS™ takes fast and easy endotoxin testing to the next level. With the MCS™, you can test five (5) samples simultaneously for endotoxin and receive results in about 15 minutes. The system works with unlicensed and licensed cartridges. Quantitative results are measured using Endoscan-V. For more information, contact your local account manager or call 1.800.762.7016 and reference item code MCS100.



LAL Workshop Registration Ongoing

The Endosafe® Summer Workshop on Bacterial Endotoxins Technology, gel-clot and kinetic methods will be held on August 21-24 in Charleston, SC. To register, please call Jill at 1.800.762.7016 or download the form from our web site, www.criver.com.





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CHARLES RIVER LABORATORIES ENDOSAFE PRODUCTS AND SERVICES NEWSLETTER


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