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*Editor's Comments*

*The appearance of a draft AAMI standard for water systems prompts a review of microbial limits for water and solutions used in hemodialysis. The AAMI document contains valuable information about individual components as well as the entire water system. Therefore, it is of interest to anyone in the pharmaceutical or medical device industry that must maintain a purified water system. Single test vials of LAL are convenient for system monitoring.*

*James F. Cooper*

The lives of countless patients with end-stage renal disease (ESRD) have been extended by dialysis procedures during the past 35 years. Approximately 250,000 Americans and about triple that number of patients, worldwide, undergo maintenance or long-term hemodialysis for ESRD. The cost of providing dialysis (artificial kidney) exceeded \$15 billion in the US during 1997. Hemodialysis is a water intensive therapy that presents an enormous challenge to produce copious amounts of high purity water, cost effectively. The occurrence of endotoxin-mediated pyrogenic reactions continues to challenge those responsible for dialysis units. This discussion focuses on voluntary water standards and ways to determine compliance with endotoxin limits.

### **Bacterial Growth and Pyrogenic Reactions**

A typical hemodialysis system consists of a water treatment system, a device for mixing water and dialysis concentrates, a distribution system and a machine to pump dialysate through the patient's dialysis unit. Water treatment must eliminate chemical toxins and harmful microbial contamination. Feed water is passed through carbon beds for dechlorination. Most dialysis centers use reverse osmosis (RO) to effectively reduce bacteria and endotoxin levels. Nevertheless, bacteria escape to downstream sites and begin to proliferate. Gram-negative bacteria (GNB) found in purified water systems include *Pseudomonas*, *Flavobacterium*, *Enterobacter* and *Alcaligenes*. All parts of the water system may become fouled with microbes in the form of biofilm, described as a collection of bacteria trapped in a gelatinous matrix that is mostly extracellular and secreted by the bacteria. Biofilm adheres tenaciously to surfaces (membranes, pipes, etc.) and is difficult to eradicate, once established. If sanitization is absent or inadequate, GNB and endotoxin (toxic byproduct of GNB) will reach dangerous levels. The only way to achieve low endotoxin concentrations is to apply periodic sanitization to prevent or limit the proliferation of biofilm inside the water system.

Investigations of pyrogenic outbreaks by the Centers for Disease Control and Prevention (CDC) always found a close association between bacterial growth and endotoxin levels. (1) Rates of pyrogenic reactions were directly related to bacterial concentration. Consequently, pyrogenic reactions were not seen when bacterial cultures were < 10 cfu/mL. The CDC has observed that pyrogenic reactions do not occur in hemodialysis units when LAL values are <5 EU/mL, but the effect of chronic exposure to low endotoxin concentrations is not fully understood.

**Endotoxin (Pyrogen).** Also known as pyrogen because of fever induction properties, endotoxin is a large molecular complex that makes up the cell wall of GNB, which is constantly shed into the environment. Endotoxin is present in municipal water in the range of 10-to-50 EU/mL. Unfortunately, it is stable, difficult to destroy by sanitizing agents, and it passes through sterilizing membrane filters. Only ultrafiltration consistently removes endotoxin.

A pyrogenic reaction is characterized by chills and fever, and may be accompanied by a fall in blood pressure and respiratory distress; it is mediated by cytokines, such as interleukin-1. Rapid increase in symptoms begins 1-to-3 hours after endotoxin exposure with peak illness occurring 3-to-6 hours afterwards. Effects of long-term pyrogen exposure are harder to define. Conditions such as  $\beta_2$ -microglobulin-associated amyloidosis and general life-shortening associated with cardiovascular disease and malnutrition are linked to chronic exposure to endotoxin. (2,3)

### Voluntary Standards for Hemodialysis Water

The European Pharmacopeia (EP) adopted an endotoxin limit of 0.25 IU/mL for water (non-mandatory) used to dilute concentrated haemodialysis fluids. Voluntary standards in the USA are less stringent. The U.S. Pharmacopeia (USP) has not addressed this issue, but features an excellent chapter on waters. (4) Of interest is a draft standard from the working group on Water for hemodialysis of the Association for the Advancement of Medical Instrumentation (AAMI). The AAMI limits for water (Table 1) are more lenient than the EP and Water for Injection, USP, which is also 0.25 EU/mL. Note that one USP-EU is equal to one EP-IU because both are referenced to the international endotoxin standard. Four dialysis-related monographs (endotoxin limits) are listed in the guidance section (non-mandatory) of the EP:

- Water for dilution of concentrated solutions (0.25 IU/mL),
- Solutions for haemodialysis (0.5 IU/mL),
- Solutions for haemofiltration and haemodiafiltration (0.25 IU/mL), and
- Solutions for peritoneal dialysis (0.5 IU/mL).

**Table 1. Microbial Limits for Water for Dialysis Applications**

Organization	Bioburden (cfu/mL)	Endotoxin (EU/mL)
European Pharmacopeia	< 100	< 0.25
EDTNA/ERCA (Proposed)	< 100	< 0.25
Japan Society for Dialysis Therapy	< 100	< 0.25
AAMI (Proposed RD62)	< 200	< 2

The limits for dialysis solutions specified in Table 1 are only representative. Renal care societies in many nations have similar microbial and endotoxin limits that apply locally. For example, the

French Health Ministry requires a limit of 0.05 IU/mL for replacement electrolyte fluids used in hemo(dia)filtration. An official Circulaire DGS/DH/AFSSAPS No. 311 (7 June 00) was recently reviewed by Gas and coworkers. (5) Many units have in-house standards that approach 0.06 EU/mL. Renal dialysis units, which generate purified water and dilute the dialysate solutions, are responsible for their own program for microbiological quality and testing.

**EDTNA/ERCA.** The European Dialysis and Transplant Nurses Association and European Renal Care Association (EDTNA/ERCA) group is developing a guideline for microbiological monitoring of water for dialysis. The EDTNA/ERCA document endorses the EP microbial limits, but goes further by addressing frequency of testing and sampling conditions. Test interval is critical because bacteria can propagate rapidly to harmful levels. Basically, this document suggests that the frequency of microbiological testing should be established and based on historical data and maintenance procedures for the water system. For example, if the disinfection interval for a system is short and test results confirm efficacy of the water quality program, a monthly test interval may be sufficient. However, if the disinfection interval is long and there is a history of elevated endotoxin levels or no existing history, then a weekly test interval is needed to assure safety. Another important aspect is that the guideline recommends having in-house capability for conducting an endotoxin test so that results of regularly scheduled tests are known quickly. In-house test capability also allows immediate investigation of solutions associated with a patient reaction so that the cause of unsafe conditions can be identified and corrected. This guideline also recommends a low-nutrient agar for monitoring bioburden.

**AAMI RD62.** The AAMI document is a comprehensive guide for water-system design, maintenance and monitoring. Valuable information and requirements are written for each component of a dialysis water system. The section for "Product Water Quality Requirements" contains microbiological limits. It states that product water used to prepare dialysate or concentrates from powder at a dialysis facility, or to reprocess dialyzers for multiple use, should contain a total viable microbial count <200 cfu/mL and an endotoxin level <2 EU/mL. However, it states that an action level for microbial count in the water shall be 50 cfu/mL and 1 EU/mL for endotoxin content. An action level is defined as the concentration of a contaminant at which steps should be taken to interrupt the trend towards higher, unacceptable levels. These limits apply to the purified water as it enters the equipment used to prepare concentrates from powder at a dialysis facility or to prepare dialysate. [The AAMI RD62 draft standard is available on CD for purchase from AAMI at the ammi.org web site. Public review of the draft is due 27MAR01.]

An appendix to the AAMI document contains rationale statements for each requirement. The scope of this standard includes equipment used to purify water for preparation of dialysate or reprocessing of dialyzers, and the devices used to store and distribute this water. The concern for reprocessing water is that bacteria would grow on the patient side of the dialyzer. Excluded from the scope of this standard are items, such as dialysate supply systems that proportion water and concentrates to produce dialysate, and systems for on-line therapy or peritoneal dialysis systems. The AAMI Committee wished more experience before dealing with on-line therapies, such as hemodiafiltration and hemofiltration, which require direct administration of large volumes of electrolytes (20-to-70 L) into blood. The latter systems depend on sequential ultrafiltration at the point of use to eliminate bacteria and endotoxins. Since these solutions are injected, the EP recommends that they be sterile and have an endotoxin limit of 0.25 IU/mL.

## **Bacterial Endotoxins Test (BET)**

Endotoxin levels are measured with *Limulus* ameobocyte lysate (LAL) reagent using procedures described in the BET section of pharmacopoeia for drugs. LAL reagents for endotoxin testing are available in single-test and multi-test forms. The single-test vial (STV) is the most suitable form for dialysis clinics because the test is as simple as adding the sample fluid directly to the test vial, which contains all of the necessary test components in a stable, freeze-dried form. A valid test requires a LAL Positive Control vial (PC); it contains LAL and a small amount of endotoxin, freeze-dried together. The PC vial is always run with a test sample to prove that no inhibiting factors are present that would inhibit the LAL test and produce a false-negative result.

The STV vial is labeled with an endotoxin sensitivity in EU/mL, that is, the least endotoxin concentration detectable by a specific reagent. As the number gets smaller, the reagent becomes more sensitive. This method is a limits test because it determines whether or not endotoxin exceeds a certain limit, rather than reveal exactly how much is present. Endotoxin measurement by single-test vials (STVs) containing LAL and LAL/positive controls is ideally suited for the dialysis clinic. The LAL test is simple and reliable when instructions are followed precisely. Required items for testing with STVs are limited to a **heating block or incubator** for undisturbed 1-hour incubation of tubes and sterile plastic or glass tubes for collection, transfer and dilution, if necessary, of water or dialysate samples.

### **Conducting LAL Tests with Single-Test Reagents**

One of the two reagents is the positive control tube (PC, red cap), which contains a mixture of LAL and endotoxin. The purpose of the PC is to confirm that there is no interference that would cause a false-negative or invalid response. For example, disturbance of tubes during incubation, residual disinfectant from sanitization procedures or high concentrations of electrolytes may interfere with the test. To assure the absence of inhibitors, 0.2 mL of water or dialysate fluid is added to the Positive Control tube (red cap). Development of an opaque gel after a one-hour incubation means that test conditions are valid. A negative result (no gel) means that inhibitors are present and that an investigation is needed to find the source of invalidity.

The dialysis assay tube (silver cap) contains LAL. Contaminated water or dialysate fluid causes the LAL to gel if the endotoxin concentration is equal to or greater than the LAL labeled sensitivity. To the LAL tube is added 0.2 mL of the same sample to determine if significant levels of endotoxin are present. A negative or no-gel result means that endotoxin content is less than the labeled sensitivity ( $\lambda$ ) of the LAL reagent. For example, if  $\lambda = 0.25$  EU/mL for the STV and the test is negative, the results are reported  $< 0.25$  EU/mL. In the case of a positive result (gel formation), the result is reported as  $> 0.25$  EU/mL, and appropriate action is taken to identify and correct the contamination.

### **Preparation of Samples for LAL Testing**

When a set of samples are ready for testing, the following steps are taken: 1) label the STVs to identify the sample being tested, and release the vacuum; 2) dispense 0.2 mL of sample into the relevant tubes, ideally in duplicate, using a 1-mL sterile syringe; and 3) mix the contents gently

and promptly incubate in a heating block or equivalent heating device. It is critical not to disturb the tubes during incubation. Acid concentrate or acetate dialysate requires dilution before testing.

After one hour, each tube is removed carefully, without breaking a potential gel, and inverted slowly and carefully toward 180 degrees. The formation of an opaque gel is a positive result. Any other result, such as a flocculent appearance, is a negative study. Policies and procedures in the clinic should dictate the frequency of LAL tests in the dialysis clinic. Ideally, water and dialysis fluids should be monitored on at least a monthly basis or when there are changes in the water treatment system or disinfection protocols. In the patient's best interest, sampling fluids close to the point of use is desirable.

An important advantage for having the ability to test for endotoxin within the renal clinic is that rapid identification of a pyrogen problem can limit the impact of a contamination event. All relevant fluids must be sampled and tested when a patient reaction occurs so that immediate corrective action can be taken. Dialysis fluid from the dialyzer (downstream side) should be collected for endotoxin analysis.

**In summary**, we have discussed pending guidelines for microbial limits in dialysis fluids. Also, a simple, quick and reliable method for endotoxin testing, using single-test vials, was described that is particularly suitable for the dialysis unit. The test can alert one to unsafe endotoxin levels and the need to take immediate corrective action for prevention of further reactions.

#### References:

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2. Dinarello CA, Kock KM, & Shaldons S. Interleukin-1 and its relevance in patients treated with hemodialysis. *Kidney Int.* **33**(Suppl. 24):S21-S26, 1988.
3. Lowrie, EG. Conceptual model for a core pathobiology of uremia, with special reference to anemia, malnourishment and mortality among dialysis patients. *Seminars in Dialysis* **10**:115-129, 1997.
4. USP 24-NF 19. <1231> Water for pharmaceutical purposes. US Pharm. Conv., Rockville. 2000, 2154-65.
5. Gas B, Goy G, & Lehuede S. Hemofiltration and Hemodialyse. *LAL Notes* (Fr.), VI.2, Automne 2000.

<b>CALENDAR</b>	
April 23	Moscow - LAL Seminar. Contact: Tandra Biomedical Services Phone: 007 0967 790531; email: <a href="mailto:tandra@online.stack.net">tandra@online.stack.net</a>
April 25	Krakow - LAL Seminar. Contact: Exna-Pol Sp. Phone: 22 37 64 30; email: <a href="mailto:exna@hotmail.com">exna@hotmail.com</a>
April 27	Athens - LAL Seminar. Contact: MetroLab Phone: 01 699 82 10; email: <a href="mailto:elifotou@metrolab.gr">elifotou@metrolab.gr</a>
May 1-2	Dublin - LAL Workshop. Contact: Medical Supply Phone: 353 1 822 4222; email: <a href="mailto:blannon@medical-supply.ie">blannon@medical-supply.ie</a>
May 3	Eindhoven, NE - LAL Seminar. Contact: Sanbio Phone: 314 132 51115; email: <a href="mailto:wvanhamond@sanbio.nl">wvanhamond@sanbio.nl</a>
August 22-25	Gel-Clot LAL Workshop, Charleston, SC. Contact: Frances Cooper Phone: 843-795-7316; fax: 843-795-7221; email: <a href="mailto:fcooper@criver.com">fcooper@criver.com</a>
August 27-30	Kinetic LAL Workshop, Charleston, SC. Contact: Frances Cooper Phone: 843-795-7316; fax: 843-795-7221; email: <a href="mailto:fcooper@criver.com">fcooper@criver.com</a>