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LAL UPDATE

Volume 6, No. 3 **September 1988**

Dear LAL User,

Plastics in the pharmaceutical industry are as ubiquitous as endotoxins. From solution containers to medical devices, plastics are not just commonplace, they are often the best material for the application. Because plastics are formed at very high temperatures that easily destroy endotoxins, plastics should be expected to be endotoxin-free. Unfortunately, the often excessive and usually uncontrolled handling of plastics prior to final packaging and sterilization practically assure their contamination with endotoxin.

LAL users' concerns with plastics fall into two categories:

1. *Direct concern. Medical device manufacturers who are interested in the contamination levels of their products.*
2. *Indirect concern. All users of LAL who employ plastic containers and transfer devices during LAL testing.*

This UPDATE, entitled "The Problems with Plastics," discusses these concerns. Chromogenic, turbidimetric, and gel-clot data from our laboratory are provided to illustrate all aspects of plastic interference in the LAL assay.

The UPDATE concludes with the regular Calendar and USP Says sections.

Sincerely,

Tom Novitsky
Editor

THE PROBLEMS WITH PLASTICS

The most common plastics encountered in the pharmaceutical industry are polypropylene, polystyrene, and polyethylene. Other plastics and variations of the above are also found quite frequently. Because of the variety of shapes, coatings, handling methods and packaging, generalizations about the types of plastics most often associated with endotoxin contamination are usually dangerous. It is the aim of this UPDATE, therefore, not to recommend or condemn a particular plastic, but rather to illustrate some of the problems encountered when extracting endotoxins from devices or containers or when using plastic components in the LAL assay.

Medical Device Testing

Section V. Medical Devices of the recent Guideline on Validation of the Limulus Amebocyte Lysate Test as an End-Product Endotoxin Test for Human and Animal Parenteral Drugs, Biological Products, and Medical Devices, lists only two requirements for a valid LAL test: 1. demonstration of LAL sensitivity, and 2. demonstration of the lack of inhibition/enhancement of LAL by the article under test. Although the FDA recognizes that endotoxin may adsorb to container surfaces (i.e. medical devices)[1], no information concerning the tendency of endotoxin to wash off these surfaces during use or testing is available. From the difficulty most people have recovering endotoxin from containers and from the lack of pyrogenic episodes traceable to single-use disposable devices (excluding dialyzers), I

believe adsorbed, non-recoverable endotoxin (using rinse recommendations in the "Guideline") in otherwise clean, sterile devices is not a health risk and need not be of concern to the device industry or the FDA.

Of more concern is the presence of LAL inhibitors in device extracts. No one is certain of the chemical nature of these inhibitors. It has been suggested that plasticizers and mold-release agents not thoroughly rinsed from a device inhibit the LAL test. Fortunately, due to the relatively large volume of liquid used to rinse, inhibition is rarely seen in devices. However, as will be illustrated below, small volume extraction of some plastic labware easily demonstrates the presence of potent inhibitors.

Plastic Labware

I discussed the adsorption of endotoxin to the surface of a variety of glass and plastic test tubes upon drying in a previous paper.[2] The following experiments conducted by Dr. Priscilla Roslansky of our staff illustrate the liquid extraction of inhibitors from plastics.

Materials and Methods

The endotoxin used was either the Reference Standard Endotoxin EC-5 (Food and Drug Administration, Office of Biologics) or ACC's Control Standard Endotoxin *Escherichia coli* O113, lot 41. Reconstituted EC-5 was stored in a Parafilm-covered 20 x 150 mm conditioned borosilicate test tube (see LAL UPDATE Vol. 3, No. 5, September 1985) at 2° - 8°C prior to use. Lot 41 was stored in its original container under similar conditions. The following LAL lots/methods were employed: Pyrotell #99-32-376GT and 99-52-395GT, kinetic turbidimetric, microplate method[3]; Pyrotell #21-02-545C, single reagent, endpoint chromogenic method[4]; Pyrotell #99-79-422, sensitivity = 0.03 EU/mL, gel-clot method.

Results

For the initial experiment, EC-5 was diluted with LRW in conditioned borosilicate glass test tubes or polypropylene centrifuge tubes and assayed turbidimetrically. The results obtained for endotoxin concentrations of 10, 1.0, 0.5, 0.25, and 0.125 EU/mL in glass were set equal to 100% for comparison with standard dilutions made using LRW in polypropylene tubes. The results are presented in Table 1. To determine whether the loss of endotoxin in the polypropylene-diluted series was due to adsorption to the plastic or inhibition from a substance(s) released by the plastic, LRW was incubated in a polypropylene tube at room temperature for 24 hrs. This water was then used to make an endotoxin standard series in glass dilution tubes. The results are presented in Table 1 under the heading "Plastic Water."

The chromogenic assay was used to confirm the inhibitory effect of the polypropylene tubes. In a second experiment an EC-5 standard was prepared in glass tubes with LRW and a standard curve generated. Optical density values obtained for 1.0, 0.5, 0.25, and 0.125 EU/mL were set at 100% for comparison with standards prepared in plastic. For this experiment LRW was stored in either polystyrene or polypropylene tubes for 1.5 hrs. This water was then used to prepare a standard endotoxin series using polystyrene or polypropylene dilution tubes, respectively. In addition, water stored in polypropylene was used to prepare a standard series in glass dilution tubes. The results are presented in Table 2.

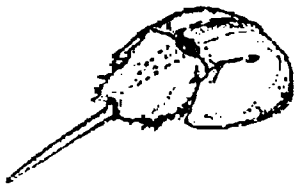

Table 1. Inhibition/adsorption properties of polypropylene in the kinetic turbidimetric method.

	Endotoxin Concentration, EU/mL				
Tube Type/Diluent	10	1.0	0.5	0.25	0.125
	Percent Endotoxin Recovered				
Borosilicate/LRW	100	100	100	100	100
Polypropylene/LRW	68	9	0	0	0
Borosilicate/Plastic Water	91	57	30	26	2

Table 2. Inhibition properties of polypropylene and polystyrene tubes using the chromogenic method.

	Endotoxin Concentration, EU/mL			
Tube Type/Diluent	1.0	0.5	0.25	0.125
	Percent Endotoxin Recovered			
Borosilicate/LRW	100	100	100	100
Polystyrene/ Polystyrene Water	92	84	60	93
Polypropylene/ Polypropylene Water	4	4	4	3
Borosilicate/ Polypropylene Water a:	47	52	38	ND
Borosilicate/ Polypropylene Water b:	39	41	32	ND

The final experiment was an attempt to eliminate the inhibitory substance in the polypropylene tubes by rinsing with LRW. Polypropylene tubes were filled with LRW and left for 2 hours at room temperature. This water was then decanted into a borosilicate glass test tube and labeled "first wash." The empty polypropylene tube was then rinsed by vortexing with three volumes of LRW. Following the final rinse the tube was filled with a half volume of LRW and allowed to stand at room temperature overnight. This last LRW fill was designated "final wash." First and final wash solutions were then used to prepare a standard endotoxin series diluted in glass test tubes. Standard series (lot 41) were also prepared in unused polypropylene tubes as well as "washed" polypropylene tubes. Results of the kinetic turbidimetric LAL analysis of these solutions are presented in Table 3. To check the effect of LAL methodology on these findings, the gel-clot method was used to assay dilutions made in glass, unused and "washed" polypropylene tubes. These data, as end points, are presented in Table 4.

Conclusions

The polypropylene tubes used in these experiments exhibited both **Adsorption** and a water-extractable **Inhibition**. The inhibitory substance(s) was equally effective in the gel-clot, turbidimetric, and chromogenic assays. The inhibitor was also fast acting. Washing the tubes eliminated some of the inhibitor but it was impossible to differentiate between adsorption and presence of additional inhibitor to explain the remaining loss of endotoxin.

The results of this study are not intended to discourage the use of plastics, specifically polypropylene, in the LAL assay. It is possible that different manufacturer's products or different lots produced by the same manufacturer vary significantly. Studies are ongoing in our laboratory to resolve this question. In the meantime, LAL users should be aware of "The Problems with Plastics" and perform some simple adsorption/inhibition tests, similar to the ones described here, prior to incorporating plastic tubes in their LAL test protocols.

It is especially important to consider the adsorption/inhibition problem in:

1. Containers used to collect or store water samples.
2. Containers used to store stock endotoxin, chromogenic substrate, and buffers or other solutions used in conjunction with the LAL assay.
3. Tubes used to make dilutions of endotoxin standards.
4. Plastic microplates or strips used to perform the chromogenic assay or microplate turbidimetric assay.

The following observations are also worth considering:

1. Some of our clients have used polypropylene tubes with no apparent adsorption/inhibition problem. Others, however, have experienced "enhancement" of the LAL assay.
2. Associates occasionally uses polystyrene dilution tubes and has experienced no problems with them. However, some of our customers, using brands of polystyrene tubes other than we use, have reported adsorption/inhibition problems.
3. Polypropylene pipet tips, which are used by Associates and by the majority of LAL users do not seem to present a problem. This is probably due to the short residence time of solutions in these tips.

Table 3. Inhibition properties of unused and washed polypropylene tubes using the kinetic turbidimetric method.

	Endotoxin Concentration, EU/mL					
Tube Type/Diluent	10	1.0	0.5	0.25	0.125	0.0625
	Percent Endotoxin Recovered					
Borosilicate/LRW	100	100	100	100	100	100
New Polypropylene/ LRW	28	14	20	34	78	ND
Washed Polypropylene/ LRW	100	66	20	30	38	67
Borosilicate/First Wash	42	26	10	14	27	66
Borosilicate/Final Wash	50	57	60	67	74	66

Table 4. Inhibition of the gel-clot LAL test using polypropylene tubes.

	Gel-clot End Point (EU/mL)					
Tube Type/Diluent	0.5	0.25	0.125	0.06	0.03	0.015
Borosilicate/LRW	+	+	+	+	+	-
New Polypropylene/ LRW	-	-	-	-	-	-
Washed Polypropylene/ LRW	+	+	-	-	-	-

References

1. Twohy, C.W. and A.P. Duran. 1986. Extraction of bacterial endotoxin from medical devices. J. Parent. Sci. Technol. *40*:287-291.
2. Novitsky, T.J., J. Schmidt-Gengenbach, and J.F. Remillard. 1986. Factors affecting the recovery of endotoxin adsorbed to container surfaces. J. Parent. Sci. Technol. *40*:284-286.
3. Remillard, J.F., P.F. Roslansky, and T.J. Novitsky. 1988. Quantitation of endotoxin using the LAL kinetic turbidimetric assay in the Titertek Twinreader. Manuscript submitted for publication.
4. Lindsay, G.K., P.F. Roslansky, and T.J. Novitsky. 1988. A single-step, chromogenic *Limulus* amebocyte lysate (LAL) assay for endotoxin. Manuscript submitted for publication.

USP SAYS

Included in Pharmacopeial Forum, July-August 1988, vol. 14, No. 4, are the following articles regarding the *Bacterial Endotoxins Test*<85> and/or the *Pyrogen Test*<151>:

1. Sterile Ceftazidine (p.3998)

Pyrogen—It meets the requirements of the *Pyrogen Test*<151>, the test dose being 1.0 mL per kg of a solution in pyrogen-free sodium carbonate solution (prepared by dissolving 9.9 g of pyrogen-free sodium carbonate in 1000 mL of Sterile Water for Injection) containing 80 mg of ceftazidine per mL.

2. Fentanyl Citrate Injection (p.4020)

Fentanyl Citrate Injection, USP XXI page 423 and page 2771 of the Seventh Supplement. It is proposed to include a *Bacterial endotoxins* test requirement to improve standards in this monograph. The limit is calculated according to the USP guidelines outlined in a *Stimuli to the Revision Process* article in *PF* 13(5) [Sept.-Oct. 1987], p. 2947.

Bacterial endotoxins—When tested as directed under *Bacterial Endotoxins Test* <85>, it contains not more than 33.3 USP Endotoxin Units per mg.

3. Indium In 111 Oxyquinoline Solution (p.4024)

Pyrogen—It meets the requirements of the *Pyrogen Test* <151>.

4. Ranitidine Injection (p.4068)

Bacterial endotoxins—When tested as directed under *Bacterial Endotoxins Test* <85>, it contains not more than 7.00 USP Endotoxin Units per mg of ranitidine.

Join us for the Annual Parenteral Drug Association Meeting

Hyatt Regency Hotel, Chicago, Illinois, October 24 - 26, 1988.

Visit our booth and plan to attend the Biotechnology Sessions on Tuesday, October 25, 1988 when Dr. Norman Wainwright, Director of Research for Associates of Cape Cod, Inc. will present **"Depyrogenation of Protein Solutions by Immobilized Endotoxin Binding Proteins Purified from *Limulus* Amebocyte Lysate."**

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