LAL UPDATE®

Volume 10, No. 3 **September 1992**

Dear LAL User:

This issue of the LAL UPDATE describes a microplate version of the kinetic turbidimetric LAL assay. Although the microplate assay should not be the method of choice for pharmaceutical quality assurance, renewed interest in clinical use of LAL has resulted in numerous inquiries for microplate method specifics. Since little has been published on the use of turbidimetric assays in microplates, this UPDATE should provide enough information for new LAL users or for those wishing to replace the chromogenic test with the kinetic turbidimetric assay. This replacement is easy since software designed for the chromogenic assay works equally well for the turbidimetric. In addition Pyrotell-T® is less expensive than the chromogenic LAL. While this paper summarizes data collected using a Titertek Twinreader, new readers with software from Molecular Devices, SLT, Anthos and Biotek are now available.

The Glucan Corner consists of an open letter by Terry Munson of CDER (published with his permission) describing the USFDA's current position on glucan contamination in pharmaceutical products.

The UPDATE closes with our regular calendar feature.

Sincerely,

Thomas J. Novitsky, Ph.D.
Editor

Quantitation of Endotoxin using the LAL Kinetic Turbidimetric Assay in an Incubating Microplate Reader

J. F. Remillard, P. F. Roslansky, and T. J. Novitsky

Recent advances in *Limulus* amebocyte lysate (LAL)-endotoxin testing have demonstrated the enhanced sensitivity and quantitation achieved with the kinetic turbidimetric method. However, due to the specialized instrumentation required for the kinetic method, the end-point chromogenic method is more widely used in the clinical laboratory. Recently, McCullough et al (1) compared microplate methods for the kinetic turbidimetric and chromogenic assay. This paper describes a turbidimetric LAL assay using an incubating microplate reader (Titertek Twinreader). We have 10 years experience with this method in our research laboratory. To perform a kinetic LAL assay, a microplate reader that provides incubation and reading capacity for 96-well microplates and an RS-232 computer interface is needed. For this application, we have written a computer program in Basic language for an Apple II GS computer. The program allows graphic representation of the optical density changes with time, generates standard curves of the log endotoxin concentration vs. log onset time as well as calculation of sample endotoxin concentration. Using LAL formulated for the kinetic method, standard curves can be generated in the range of 0.01 to 100 EU/mL. Coefficients of variation for replicates of the endotoxin standards tested averaged 11.1%. Sample volumes of 0.1 mL can be successfully assayed. Data are presented on water, saline, human serum albumin, human amniotic fluid, and cotton lint extract samples.

Introduction

A kinetic turbidimetric method using *Limulus* amebocyte lysate (LAL) for the determination of endotoxin concentration in pharmaceuticals, biologicals and medical device products has been developed (2, 3, 5, 7, 8, 9, 10, 11). Unlike end-point methods (gel-clot, chromogenic and turbidimetric), determining endotoxin concentrations using the kinetic turbidimetric method is accomplished by measuring the rate of the reaction or the

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amount of time required for the reaction to reach a specified optical density (O.D.). This allows the user to span up to five orders of magnitude in endotoxin concentrations rather than diluting to the narrow sensitivity range of an end-point assay (chromogenic or turbidimetric). This range in sensitivity allows determination of endotoxin concentrations in products with a greater degree of quantitation than previously available and readily assesses sample inhibition and enhancement effects relative to water.

The utilization of an LAL kinetic test, however, requires instrumentation capable of multiple readings at equal intervals and incubation at the standard temperature of 37°C. Computer software is required to collect data, plot O.D. changes vs. time and calculate the time required to attain a predetermined milliabsorbance level (Time of Onset or T_o). Onset time data are regressed against a series of endotoxin concentrations to determine the equation of the standard line and the correlation coefficient. Onset times of samples are then compared to this standard line. Many commercial programs which are capable of these operations are now available. In fact, because the guidelines for kinetic LAL assays are now the same for chromogenic turbidimetric methods, a program designed for use with the chromogenic test will also work for the turbidimetric method.

Although there now exists instrumentation specifically designed to perform the LAL kinetic turbidimetric assay, microplate technology is already well established in the clinical and research laboratory. The Titertek Twinreader is an instrument which will incubate and read a multiwell plate at predetermined time intervals. Raw optical density data can be transmitted from an RS-232 serial port to an external computer. We report here the computer/software interfacing required for microtiter plate readers to perform the kinetic turbidimetric assay as well as the endotoxin analyses of several product samples using the plate reader. These samples (water, saline, normal serum albumin, human amniotic fluid, and cotton lint extract) represent the types of LAL-endotoxin reactions which are characteristic of the kinetic turbidimetric assay.

Materials and Methods

Reagents and glassware have been described in detail (7, 8). LAL reagent water (LRW) was purified by distillation (6). Control standard endotoxin was prepared from *E. coli* O113 lipopolysaccharide (ACC lot #29). The LAL (ACC lot #99-01-347-GT and ACC lot #99-23-368-T) was formulated for the kinetic turbidimetric assay. Standard curves were generated from six to ten twofold dilutions of endotoxin ranging within the limits of 100 EU/mL to 0.01 EU/mL. The standards (0.1 mL) were added in duplicate in Rows A and B of a certified endotoxin and β-glucan free, 96-well flat bottom microplate, Pyroplate™. Sample and/or sample dilutions (0.1mL) were added in duplicate or triplicate to remaining wells. Standards and samples were then assayed by adding 0.1 mL of LAL using a repeating pipetter. The plate was shaken for 1 minute using a plate shaker and placed in an incubating plate reader (Titertek Twinreader).

Data are sent from the plate reader via an RS-232C serial interface port to an Apple II GS computer. A Basic program, Twinreader Apple Program (TAP), was written which collects

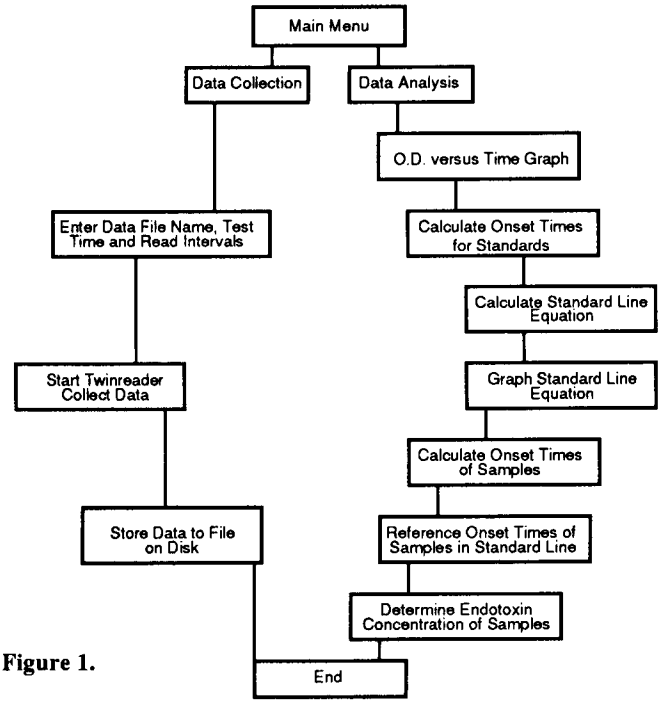

Figure 1.

the absorbance data sent from the Twinreader at predetermined time intervals of two minutes for one hour. A flow diagram of the program is shown in **Figure 1.** During Data collection, the first absorbances sent at T=0 minutes are adjusted to baseline. Subsequent changes in the absorbance values are then stored to disk. In Analysis, TAP retrieves the data file from the disk and plots the change in optical density with time for each standard endotoxin concentration and sample. An optical density abscissa is selected where the slope is constant over several read intervals for all of the endotoxin concentration standards. 50 mA has been routinely used and found to yield consistent results. Onset times (T_o) are determined by linear regression of three optical density values above and below the optical density abscissa. A log T_o vs. log endotoxin concentration regression line equation is calculated in addition to a correlation coefficient (12). Onset times of samples and/or sample dilutions are referenced to the standard line to determine endotoxin concentrations.

Water, or those products which react like water, can be assayed directly against a water standard. Products which enhance or inhibit the LAL reaction can be tested using either of the two methods outlined in the FDA Guideline (4). The product can be diluted in LAL reagent water (LRW) until the response of the LAL-product-endotoxin reaction is equivalent to LAL-water-endotoxin reaction. This is termed the Water Equivalent Dilution (WED) and is determined by comparing endotoxin spike recoveries in the test sample against spike recoveries made in LAL reagent water. Endotoxin spiked products which enhance/inhibit the LAL assay will attain onset time sooner/later than the equivalent spike made in water. Alternatively, a range of endotoxin standards can be made in the product or product dilution itself, and a Product Standard Curve (PSC) generated. PSC's define the LAL response in the product and avoid inhibition/enhancement effects caused by referencing a water standard.

Reproducibility of the LAL kinetic turbidimetric method in the Twinreader was determined by assaying twenty replicates of five endotoxin concentrations: 100, 10, 1, 0.1 and 0.01 EU/mL. Replicates for each endotoxin concentration were assayed near the center of the multiwell plates in columns 4, 5, 6, 7 and 8 of rows C, D, E, and F. Means, ranges and coefficients of variation of onset times were calculated for each endotoxin concentration. Means, ranges and coefficients of variation for endotoxin concentration were calculated by referencing onset times against a representative water standard curve.

Results

Kinetic curves for endotoxin concentrations (10 EU/mL-0.3 EU/mL) in LRW are shown in **Figure 2.** The curves show a stable baseline representative of 100% transmission followed by a rapid onset in turbidity resulting from the LAL-endotoxin reaction. The time required for the development of turbidity is inversely proportional to endotoxin concentration. The greater the endotoxin concentration the sooner the onset of turbidity and the steeper the slope. Maximum O.D. is not proportional to endotoxin concentration and so is not a reliable indicator of contamination levels. In **Figure 3,** log onset times, determined at 50 mA, were calculated for the data from **Figure 4** and regressed against the log of the endotoxin concentration.

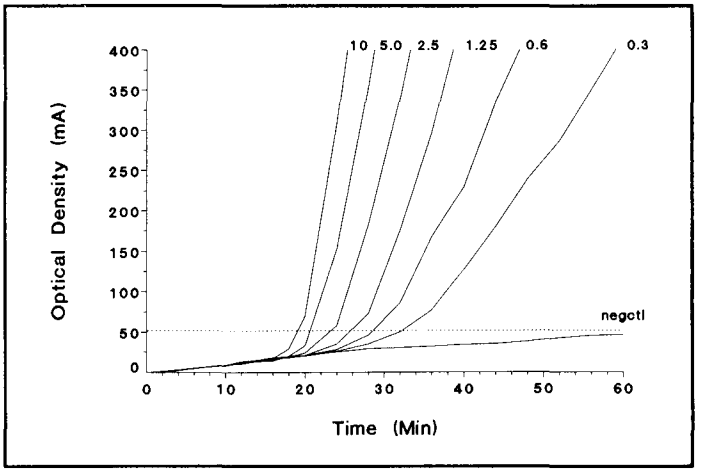

Figure 2.

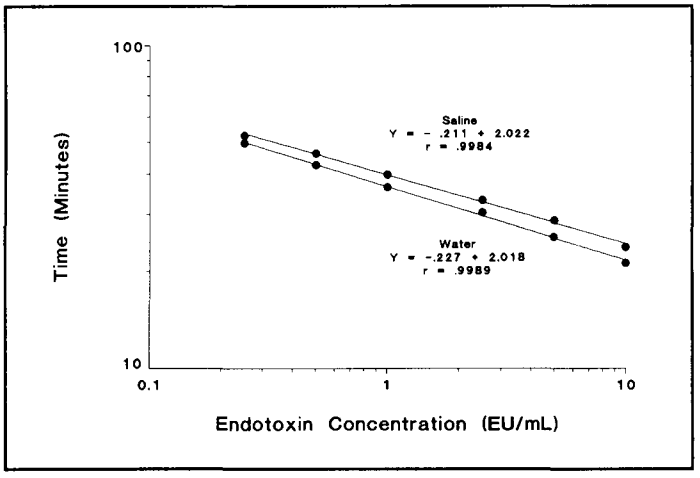

Figure 3.

In **Figure 4,** kinetic data are compared for three endotoxin concentrations in 0.9% saline and water. Each endotoxin concentration in saline reacts slower and has a greater T_0 than the corresponding endotoxin concentration in water. Thus, when these data are plotted as log T_0 vs. log endotoxin concentration (**Figure 3**), the saline standard curve is above the water standard curve demonstrating the inhibitory effect of saline.

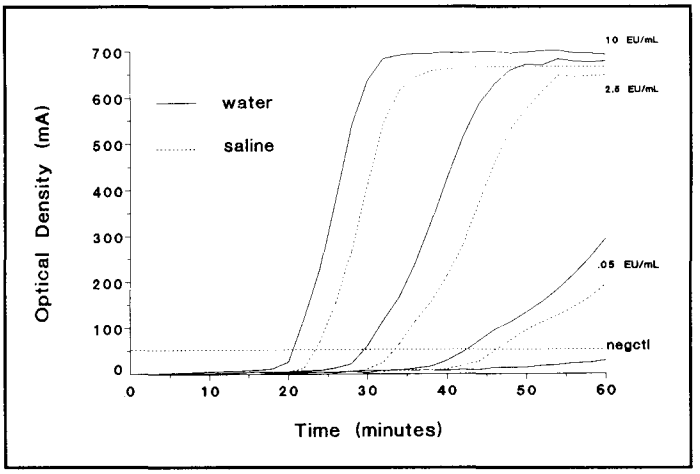

Figure 4.

In **Figure 5,** a sample of human serum albumin (HSA) was diluted in water. Each dilution and a water control were spiked with 1.0 EU/mL of endotoxin. At a 1:10 and 1:20 dilution, the reaction was inhibited relative to the water spiked with 1.0 EU/mL, while at 1:100 and 1:200 there was an enhanced reaction. At a dilution of 1:1000, the kinetic curve of HSA closely approximates the water curve and the resultant T_0 is nearly equivalent to the 1.0 EU/mL spike in water. Thus, at a dilution of 1:1000 or greater, inhibition/enhancement relative to water had been virtually overcome and the sample's endotoxin concentration can be determined using a water standard curve. In this figure, it is clear that the total amount of turbidity in the samples was depressed in the 1:10, 1:20, 1:100 and 1:200 dilutions of HSA. This effect is eliminated at the 1:1000 dilution.

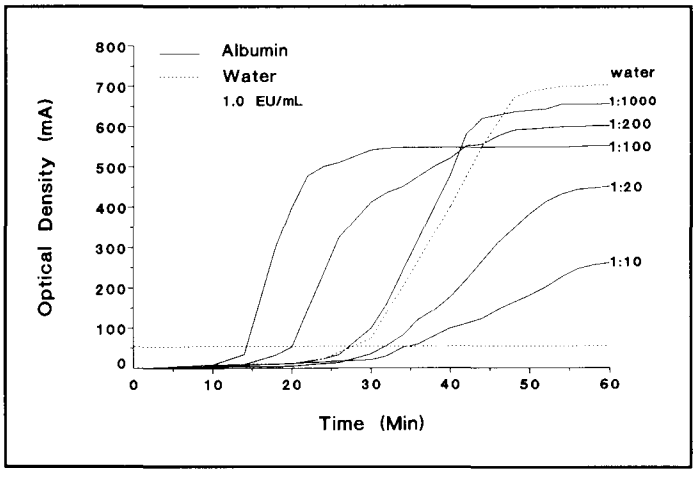

Figure 5.

In **Figure 6**, a sample of human amniotic fluid was diluted in water. Each dilution was spiked with 1.0 EU/mL and compared to the same spike made in water. Unlike human serum albumin, amniotic fluid did not exhibit inhibition. It did, however, show enhancement and a lower maximum O.D. which was overcome at a dilution of 1:40.

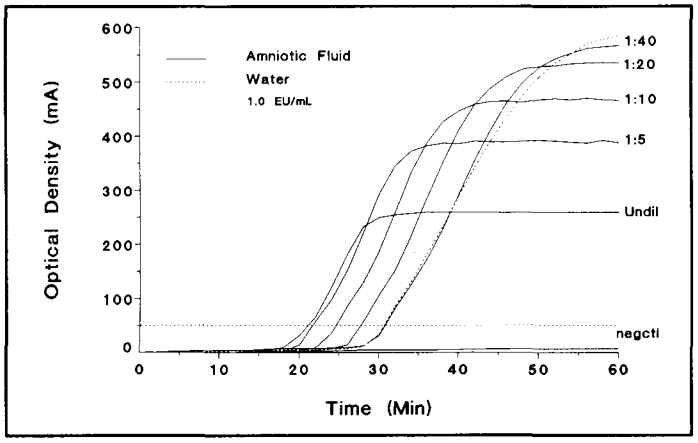

Figure 6.

Table 1 shows the results of testing water extracts of cotton lint. The high concentration of endotoxin in these samples required large dilutions, thus eliminating any observable inhibition or enhancement.

Table 1. Endotoxin Concentration of Cotton Lint Extracts in µg/g (ppm).

Sample	Dilution	Endotoxin Concentration
1A	100X	50
1B	100X	58
2A	1000X	132
2B	1000X	110
3A	1000X	89
3B	1000X	87

Extraction: 20 mg cotton lint in 10 mL of LRW was continuously shaken at room temperature for one hour.

Table 2 shows the onset time and endotoxin concentration means, ranges and coefficients of variation for twenty replicates of five endotoxin concentrations. Coefficients of variation ranged from a low of 5.9% for 100 EU/mL to 21.1% for 0.01 EU/mL.

Table 2. Reproducibility of the Kinetic Turbidimetric Method

Endotoxin Conc.	Linear Onset Time (min.)			Measured Endotoxin Concentration		
	Mean	Range	C.V. (%)	Mean (EU/mL)	Range (EU/mL)	C.V. (%)
0.01 EU/mL	65.1	60.0 - 67.8	4.2	0.01	0.009 - 0.013	21.1
0.1 EU/mL	42.9	41.5 - 43.2	1.1	0.1	0.84 - 0.107	6.1
1.0 EU/mL	25.4	24.7 - 26.1	1.5	1.1	0.87 - 1.16	9.7
10.0 EU/mL	17.8	16.9 - 18.3	2.5	10.8	8.5 - 12.1	12.9
100.0 EU/mL	14.4	14.1 - 14.9	1.2	92	78.5 - 110.	5.9

Discussion

The kinetic turbidimetric method is a quantitative and sensitive test for the analyses of endotoxin in pharmaceuticals, parenterals, biologicals and devices (3, 7, 9, 10). Since the LAL-endotoxin reaction is assayed throughout test incubation, the narrow detection window dictated by end-point or fixed incubation time methods such as the gel-clot, chromogenic or end-point turbidimetric is avoided. Detection sensitivities are enhanced since onset time determinations are dependent on the initial turbidity formed during LAL-endotoxin reactions rather than gel formation. Moreover, the test is simple to perform requiring only the addition of lysate to the sample rather than the multiple steps necessary for other assays.

The Titertek Twinreader has been shown to be a suitable instrument for use in the analysis of endotoxin using the LAL kinetic turbidimetric method. The instrument incubates microtiter plates at 37°C and is capable of read intervals as short as one minute. Although other instruments exist which are dedicated to performing the kinetic turbidimetric assay such as the Wako Toxinometer (Wako Pure Chemical Industries) and LAL-5000 (Associates of Cape Cod), if the user is willing to accept reduced precision (due to poor temperature control and addition timing errors) and increased false positives (due to plate/well contamination of uncertified plates), microtiter plate readers are versatile instruments and are already available in many laboratories. The software, available for the APPLE II GS, performs the necessary functions of data capture and analyses.

The LAL-endotoxin-product reactions generated in the plate reader were characteristic of the types of kinetics seen when using the kinetic turbidimetric method with other instruments. Enhancement is observed when endotoxin spiked samples (product positive control) achieve onset time faster than the equivalent spike in water (water positive control). This was seen in HSA samples at the 1:100 and 1:200 dilutions. Inhibition is a delay in onset time in spiked samples relative to water as observed at the 1:10 and 1:20 dilutions of HSA. At sufficient dilutions the LAL response to a given endotoxin concentration in a sample is equivalent to that made in water. At this WED, the unspiked sample may be analyzed relative to a water standard. The WED was attained in the HSA and the amniotic fluid samples at dilutions of 1:1000 and 1:40, respectively. Analysis of complex samples such as HSA and amniotic fluids, which demonstrate dilution dependent inhibition and enhancement, can be confusing when using end-point or fixed time assays.

An alternative to using a water standard curve consists of analysis of samples against a product standard curve such as the 0.9% saline standard. PSC's, however, necessitate product which contains considerably less endotoxin than the pass-fail limit for that product. Using clean product for standard curves is required to avoid endogenous background contamination from influencing the onset times of the endotoxin standards.

Although the Twinreader is an adequate instrument for testing endotoxin contamination in samples using the kinetic turbidimetric method, several precautions must be considered. Unlike the dedicated instruments mentioned above which use disposable borosilicate glass tubes, the Twinreader utilizes plastic microtiter plates. Since plastics may interfere with the

LAL assay, plates should be carefully checked prior to rountine use. However, use of certified microplates such as Pyroplate can eliminate these problems. In addition, LAL must be added to all of the wells of the plate before it is shaken and placed in the instrument. Thus, onset times may be offset or delayed due to the order in which the lysate was added to the wells. This error is largely negated when repeating pipetters are used to dispense the LAL. In contrast, each tube used in dedicated systems is individually mixed, initiated, timed and read. A further consideration when using microtiter plates for LAL testing is the wells located at the plate edges. These wells can be easily contaminated during handling. They may also not heat at the same rate as the wells in the center of the plate. Therefore, it is recommended that these wells be avoided if possible, (i.e. 60 wells are useable).

The range and coefficient of variation data for the onset times and corresponding endotoxin concentrations showed, in general, no concentration dependent variability within the range tested. At high endotoxin concentrations (>10 EU/mL) turbidity increases more rapidly after the initial lag than at the lower endotoxin concentrations. Thus the O.D. versus time kinetic lines are well defined and intersect the onset time abscissa (50mA) at an acute angle. Above 100 EU/mL there is very little separation between twofold dilutions of endotoxin standards and resolution is degraded. Lower endotoxin concentrations possess less slope and so do not intersect the onset time abscissa acutely. However, this decrease in slope is countered by increasingly longer time intervals between endotoxin concentrations thus increasing resolution. Coefficients of variation increase at very low concentration (0.01 EU/mL) since slopes become extremely attenuated and O.D. development is not significantly above baseline. Regardless of the endotoxin concentration it is recommended that all standards and samples are assayed in at least duplicate.

Given the precautions and variability discussed above, the data demonstrate that good LAL turbidimetric kinetics can be achieved in an incubating plate reader for a wide range of samples. Although a dedicated system, such as the LAL-5000, is better suited to the kinetic turbidimetric method and is not subject to the limitations and precautions mentioned above, the plate reader has been found to be an acceptable alternative.

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Calendar

PDA -Annual Vendors Night
Marriott Hotel - Cambridge, MA
October 21, 1992

Parenteral Drug Association Meeting
Hyatt Regency Embarcadero
San Francisco, CA
November 11-13, 1992

BioEast '93
Omni Shoreham, Washington, DC
January 24-28, 1993

Glucan Corner

Statement concerning Glucans and LAL-Reactive Material in Pharmaceutical and Medical Devices

From: Terry E. Munson, Chairman, FDA LAL Task Force

To Whom It May Concern:

It has been reported to the LAL Task Force that information has been circulated concerning FDA's position on glucans and LAL-Reactive Material (LAL-RM) in pharmaceuticals and medical devices. Glucans are defined as polysaccharides composed only of recurring units of glucose, such as, glycogen, starch and cellulose.

FDA is not aware of data indicating that glucans or LAL-RM are common in pharmaceuticals or may be common in medical devices or whether they may pose a health hazard to patients using these products. The only product that has been shown to contain LAL-RM is dialysis membranes made of cellulose. LAL-RM is composed of very small fragments of the cellulose which break loose from the filter matrix. Studies in animals and tissue cultures indicate that LAL-RM is non-toxic in the quantities detected in dialyzers. Some investigators have indicated that LAL-RM may be responsible for some of the adverse reactions seen in dialysis patients. However, no direct correlation has been established. LAL-RM is the only glucan that has been reported in any medical device. LAL-RM is not considered a contaminant, because its source is not extrinsic to the device. It is a breakdown product of the cellulose membrane that is a component of the device. Since the majority of medical devices do not contain natural materials, there is no source of glucans in these devices.

No cases of glucans or LAL-RM have been reported in parenteral drug products. Most parenteral drug products are manufactured from chemically synthesized components. This fact coupled with good manufacturing practices makes the possibility of contamination with glucans very remote. It appears that large amounts of glucans are required (at least 1,000 times more by weight than endotoxin) to elicit a LAL positive reaction.

At this time, FDA considers that the presence of glucans in parenteral drug products and most medical devices to be more of a theoretical than actual problem. Firms should be aware that false positive results may be possible when testing medical devices having cellulose based components. It is the responsibility of the manufacturer to conclusively establish that any positive LAL test is not due to endotoxin contamination. FDA will consider whether parenteral drug products or medical devices are adulterated due to the presence of glucans on a case-by-case basis, until such time as more information is obtained.

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