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COMPARISON OF PLASMA EXTRACTION TECHNIQUES IN PREPARATION OF SA--ETC(U).
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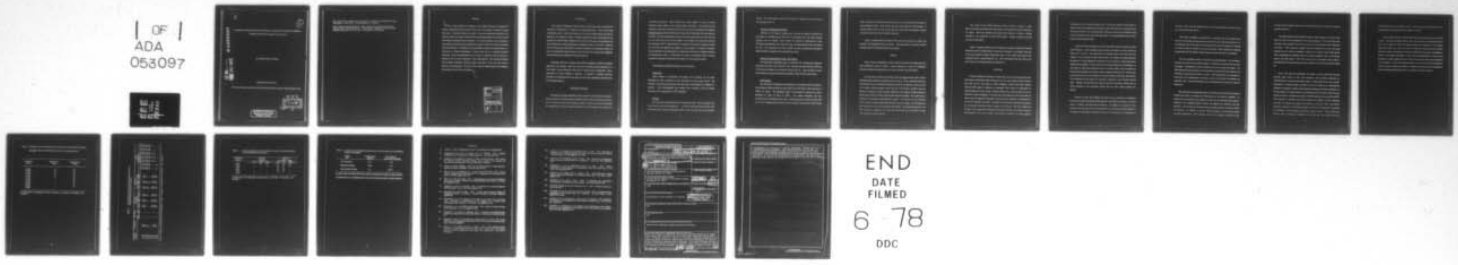

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Comparison of Plasma Extraction Techniques in Preparation of Samples for Endotoxin Testing by the Limulus Amoebocyte Lysate Test

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The views of the authors do not purport to reflect the positions of the Department of the Army or the Department of Defense.

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Abstract

Due to the presence of inhibitory and possible mimicking substances in plasma, difficulties have occurred in the use of the Limulus Amoebocyte Lysate (LAL) test. Currently, there are a variety of extraction techniques discussed in the literature which are used to remove these interfering substances, but there is little information comparing these techniques. Five such procedures were compared in their ability to provide an extracted plasma sample in which low levels of endotoxin could be indicated. In addition, they were judged on their ease of performance in a laboratory or clinical environment. Further study led to a modified extraction technique. With this modification the LAL test can indicate levels of endotoxin in plasma as low as can be indicated in water (0.06 ng/ml). This modified technique also included procedures whereby possible mimickers of the LAL test found in plasma were inactivated. The use of this technique should improve the reliability and validity of the LAL test in plasma.

Introduction

The Limulus Amoebocyte Lysate (LAL) test has been used to indicate the presence of endotoxins in a variety of samples (8,12,14,21). The test has been successfully used in such body fluids as urine and cerebral spinal (4,12,17,21). However, the testing of serum or plasma samples has been complicated by the presence of proteins which inhibited the test (15), as well as by the possibility that some substances in plasma might mimic endotoxin in the LAL test (6). As pointed out by Tuazon et al., the need for chemical alteration of the plasma sample may alter the sensitivity and/or accuracy of the test and may explain the ambiguities among studies for the detection of endotoxemia (21).

Currently, there are a variety of procedures designed to prepare the plasma sample for LAL testing. There are little data comparing these procedures as to their effect on the sensitivity of the test or to their ease of application. Such a comparison of these methods is reported. In addition a modified extraction procedure is introduced which may help to remove the ambiguities associated with LAL testing in plasma.

Materials and Methods

All plasma extraction procedures were compared in their ability to provide a plasma sample in which low levels of added endotoxin could be detected by the LAL test. To ensure that the level of plasma inhibitor would be the same when making the comparisons, the same pooled endotoxin-free human plasma was used in all

extraction procedures. Each method was tested against a series of known endotoxin levels added to the plasma before extraction. The lowest level of endotoxin which gave a solid gel in the LAL test (4+) was considered the endpoint. Escherichia coli endotoxin (Associates of Cape Cod, Woods Hole, MA) was used as a standard. Pyrotell (Associates of Cape Cod, Woods Hole, MA) LAL was used in a 0.1 ml volume with 0.1 ml of test sample. All readings were made after one hour of incubation at 37°C. Determination of reaction in the test was made by inverting the test tube 180 degrees and looking for gel formation or change in viscosity. Lysate sensitivity was determined by testing serial dilutions of standard endotoxin in endotoxin-free water. To determine the effect of phosphate buffer on lysate sensitivity, endotoxin standard was also diluted in 0.5M phosphate buffer.

The following extraction procedures were compared:

Chloroform

Equal volumes of chloroform and plasma were vortexed for two hours following the basic procedure as first described by Levin et al. (14,15). The emulsion of chloroform and plasma was separated by centrifugation at 1000g for 10 minutes. After centrifugation test samples were withdrawn and any residual chloroform was evaporated in a 37°C waterbath.

pH Shift

The procedure of Reinhold and Fine was followed (18). Plasma samples were first lowered to pH 4.0 with glacial ac .ic acid and then returned to pH 6.0 with anhydrous dibasic potassium phosphate buffer. Plasma samples were then tested at

pH 6.0. To ensure optimum pH for the reaction, an additional test was set up for each sample at pH 7.0.

Dilution in Endotoxin-Free Water

Inhibitors in the plasma samples were removed by dilution, following the procedure of Goldstein et al. (10), with the exception that the diluted plasma samples were not heated. Each sample was diluted in endotoxin-free water (Travenol, Deerfield, IL) 1:10, 1:20, and 1:100. All three dilutions were then tested by LAL. This procedure was later modified by using endotoxin-free phosphate buffer (0.5M) in the place of the endotoxin-free water.

Dilution in Endotoxin-Free water with Heating

The procedure of Goldstein et al. was followed (10). Samples were diluted in endotoxin-free water 1:10, 1:20, and 1:100. Samples were then heated to 95°C for 10 minutes and allowed to cool before testing with LAL. This procedure was also later modified by using endotoxin-free phosphate buffer (0.5M) as the diluent.

Gel Filtration

The procedure of Hollander and Harding (11) was followed with the exception that phosphate buffer (0.5M) was used rather than 0.5M NaCl, which was found to inhibit our lysate. The phosphate buffer was prepared by heating the sodium phosphate at least 12 hours at 190°C. The sodium phosphate was then reconstituted with endotoxin-free water. Bio-Rad glass barrel econo-columns (50X 0.7 cm, 737-1262)(Rockville Center, NY) were packed with Bio-Gel P-200, 50-100

mesh. Columns were rendered endotoxin-free by overnight flushing with endotoxin-free phosphate buffer. Void volume (V_o) was determined with blue dextran. A sample volume of 0.2 ml was run through the column and the endotoxin fraction was found in the first 1.2 ml of effluent after V_o.

Using a standard biuret procedure (19), the level of protein in certain test samples was determined after extraction. This information was used to further evaluate some of the above extraction procedures.

Results

Table 1 shows a comparison of lysate sensitivities when the endotoxin sample was contained in water or buffer. Lysate sensitivity in water was 0.06ng/ml, whereas in buffer the sensitivity for the same endotoxin was 0.006 ng/ml.

As can be seen in Table 2, the lowest level of E. coli endotoxin which could be indicated after chloroform extraction was 0.2 ng/ml. At the endotoxin levels used, the pH shift was not an effective method for removal of inhibitors. Even at a level of 5.0 ng/ml, neither samples at pH 6 nor pH 7 could elicit a positive LAL test. Dilution of plasma in water was also ineffective (> 0.6 ng/ml), though heating the sample after dilution to 95°C improved the results (0.2 ng/ml). This method was as effective as chloroform extraction (0.2 ng/ml). The lowest level of endotoxin which could be indicated after gel filtration of plasma samples was 0.04 ng/ml and near solid gels of LAL (+3) occurred at 0.02 ng/ml. This represented nearly a 10-fold increase in sensitivity over chloroform extraction and the dilution in water with subsequent heating method.

The results of the modified dilution method are shown in Table 3. When plasma was diluted in buffer rather than water, a solid gel reaction (4+) occurred at 0.6 ng/ml. When the samples were heated after dilution in buffer, a 10-fold increase in the sensitivity occurred (0.06 ng/ml). Partial reactions (+2) also occurred at 0.04 ng/ml.

Table 4 compares protein levels in endotoxin end-point samples from the gel filtration and the modified dilution extraction techniques. Less protein was found in the gel filtration sample (0.4g/100ml) than in the sample extracted by the modified dilution method (0.6g/100 ml). Both represented less than 10% of the protein found in unextracted plasma (7.2g/100 ml).

Discussion

Though chloroform extraction of plasma may not be the only source of error affecting the sensitivity and/or accuracy of the LAL test in plasma, the fact that it has been used in most cases in which ambiguous results have been obtained (5,9,13,14,20) makes it essential to investigate other means of preparation of plasma samples for LAL testing. Results presented here indicate that it is not as effective as other techniques. It is quite time consuming and care must be taken to ensure that the degree of vortexing is the same for all samples, or differences in the level of inhibitor will result. In addition the effect that chloroform extraction has on possible mimickers found in plasma is not known. This may explain the findings of two groups of investigators (3,9) using chloroform extraction, who have found positive LAL test in either non-critically ill patients or human subjects

considered to be of a normal healthy state. It might also explain the association of positive tests with fungal (9,20) and gram positive (20) infections, though there has been some evidence of an endotoxin-like substance isolated from pathogenic fungi (1), and of positive LAL tests with high levels of the peptidoglygan found in gram positive micro-organisms (22).

Of the extraction procedures tested, the pH shift method was found to be the least effective. At the endotoxin levels tested, no positive LAL test occurred at either pH 7 or pH 6. This procedure was repeated many times with the same results. It is not known if these results reflect the difficulty of performing this procedure or if they represent the inability of this technique to sufficiently remove inhibitors from plasma. In any case its use in clinical studies may be questioned. In one report in which this method was used in a clinical study, poor correlation was found between positive LAL tests and gram negative infections (16). Those results may have been due to the ineffective removal of plasma inhibitors, which seemed to be indicated by the report of a decrease in gelation in the extraction control tube. Feldman and Pearson (7), using the pH shift extraction method, did not report problems in the extraction control tube but their clinical results were similar.

Dilution in water plus heating was found to be as effective as chloroform extraction, though it has several advantages over chloroform extraction. It is much easier to perform and less time comsuming. The degree of extraction of two samples from the same source can be assumed to be constant, whereas variation in the degree of chloroform extraction can occur if the intensity of vortexing is not

the same. Also, it has the added feature of inactivating possible mimickers of the LAL test found in plasma (10).

The effect of heating on inactivation of inhibitors with this method can be seen in the comparison between dilution and dilution with heating (see Table 2). At an endotoxin level of 0.6 ng/ml a weak positive reaction (2+) resulted at a dilution of 1:100; no positive reaction occurred at 0.2 ng/ml. When heating was included, strong positives (+4) resulted at these endotoxin levels with only a 1:10 dilution.

The most effective means of extraction was gel filtration. This procedure, however, has several serious disadvantages. It is difficult to perform and very time consuming. Rendering the column endotoxin free is not always successful. The effectiveness of this procedure is very dependent on the nature of the column, with variations occurring from column to column. Most importantly, this procedure is costly due to the requirement of testing a number of fractions to determine the endotoxin zone. These disadvantages make it inadvisable for wide use in a clinical laboratory.

The substitution of phosphate buffer as the diluent in the dilution and heating method was found to increase the effectiveness of the dilution technique. As measured by the protein in the samples and the level of endotoxin which could be indicated, this modified dilution method was slightly less effective than gel filtration. It is, however, a much less time consuming and more consistent method of extraction. In addition, it has the added advantage of inactivating possible mimicking substances. Since protein was found in samples extracted by gel

filtration this technique would need to include heating of the fractions to remove possible mimickers.

The effectiveness of this modified dilution method appears to be due to the effect of buffer on the sensitivity of the lysate employed. It was found that if the endotoxin was contained in buffer there was a 10 fold increase in the sensitivity of the lysate. Thus, using the modified dilution extraction, the LAL test could indicate as low a level of endotoxin in plasma as it could in water (0.06 ng/ml). When considering the dilution factor the actual endotoxin level in the diluted plasma was 0.006 ng/ml. This is the lowest level of endotoxin which could be indicated (+4) by this lysate system when endotoxin was diluted in buffer (see Table 1).

Due to the ease and consistency with which it can be performed and the increase which occurs in LAL test sensitivity, this procedure represents an improvement over the other procedures tested. Cooperstock (2) has used a similar procedure in a clincical study and found it to be very effective in indicating the presence of endotoxin in plasma from patients suffering from Reye's syndrome. In that procedure the buffer (tris) was incorporated with the lysate rather than the sample and only a 1:3 dilution was used. In addition samples were heated at 100°C. This decrease in dilution may further improve the ability of this extraction procedure to indicate low levels of endotoxin by the LAL test. Any further decrease in dilution of the plasma sample may result in precipitation of proteins and endotoxin during heating causing false negative tests. Their results also indicate that an increase in sensitivity of the LAL test may result if the LAL

reaction takes place in a buffered system. Thus, all procedures using the LAL test could benefit by the incorporation of buffer in the test.

To summarize, the LAL test has been used to detect endotoxemia with varied results. The present findings indicate that some of these inconsistencies may be due to the method in which the plasma sample was prepared for LAL testing. Improved results may occur by using an extraction procedure which involves dilution in buffer and heating to inactivate inhibitory and possible mimicking substances. This report also points to the need for standardization of the LAL test procedure in plasma before the true worth of the test for indicating endotoxemia can be judged. It is felt that standardization in not only the extraction method but also the Limulus lysate and endotoxin standard used would make comparisons of clinical studies possible and improve the validity of the test in plasma.

Table 1. Comparison of the sensitivity of Pyrotell lysate when standard endotoxin (E. coli) is diluted in endotoxin-free water or phosphate buffer.

Endotoxin ng/ml	Endotoxin in water	Endotoxin in buffer
0.100	4+	4+
0.080	4+	4+
0.060	4+	4+
0.040	1+	4+
0.020	1+	4+
0.010	-	4+
0.008	-	4+
0.006	-	4+
0.004	-	2+
0.002	-	1+
0.000	-	-

4+=solid gel; 3+=running gel; 2+=gel clumping; 1+=increase in viscosity; -=no reaction

Table 2. Comparison of the extraction techniques used to remove inhibitors found in plasma.

Endotoxin ng/ml	Chloroform	pH shift		Dilution in water			Dilution in water + heat			Gel filtration fraction #						
		ph6	ph7	1:10	1:20	1:100	1:10	1:20	1:100	6	7	8	9	10	11	12
5.00	NT	-	-	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
1.00	NT	-	-	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
0.60	4+	-	-	-	1+	2+	4+	4+	-	NT	NT	NT	NT	NT	NT	NT
0.20	4+	-	-	-	-	-	4+	2+	-	NT	NT	NT	NT	NT	NT	NT
0.08	-	-	-	-	-	-	-	-	-	-	-	1+	4+	1+	-	-
0.06	-	-	-	NT	NT	NT	NT	NT	NT	-	-	1+	4+	1+	-	-
0.04	NT	-	-	NT	NT	NT	NT	NT	NT	-	-	1+	4+	1+	-	-
0.02	NT	-	-	NT	NT	NT	NT	NT	NT	2+	3+	-	-	-	-	-
0.00	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

NT=not tested; 4+=solid gel; 3+=running gel; 2+=gel clumping; 1+=increase in viscosity; -=no reaction

Table 3. Limulus Amoebocyte Lysate reactions in plasma samples extracted by the Modified dilution method.

Endotoxin ng/ml	Dilution in buffer			Dilution in buffer + heat		
	1:10	1:20	1:100	1:10	1:20	1:100
0.60	-	2+	4+	4+	4+	3+
0.20	-	-	1+	4+	4+	2+
0.08	-	-	-	4+	4+	-
0.06	-	-	-	4+	2+	-
0.04	-	-	-	2+	2+	-
0.00	-	-	-	-	-	-

4+=solid gel; 3+=running gel; 2+=gel clumps; 1+=increase in viscosity; -=no reaction

Table 4. Protein Level in Samples Extracted by the Gel Filtration and the Modified Dilution Techniques.

Sample ID	Protein Level g/100ml	% Protein of Unextracted Sample
Gel Filtration	0.4*	5.6
Modified Dilution	0.6#	8.3
Unextracted Plasma	7.2	100.0

*protein level in fractions #10 from samples containing 0.04 ng/ml E. coli endotoxin.

protein level in 1:10 dilution from sample containing 0.06 ng/ml E. coli endotoxin.

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of performance in a laboratory or clinical environment. Further study led to a modified extraction technique. With this modification the LAL test can indicate levels of endotoxin in plasma as low as can be indicated in water (0.06 ng/ml). This modified technique also included procedures whereby possible mimickers of the LAL test found in plasma were inactivated. The use of this technique should improve the reliability and validity of the LAL test in plasma.