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Interference in the limulus amebocyte lysate assay for endotoxin determination in peritoneal dialysis fluids and concentrates for hemodialysis

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Abstract

The interference of the saline concentration of fluids for peritoneal dialysis and concentrates for hemodialysis on the limulus amebocyte lysate (LAL) assay for endotoxins was investigated. The experiments were carried out individually with each substance that compose fluids for hemodialysis, to determine the possible inhibition or enhancement effects that they could cause on the LAL assay. The compositions were also assayed to investigate the possibility of synergistic effect. They were assayed by the gel-clot method from two different suppliers, and the samples that showed inhibition effect were also assayed by the chromogenic method. The samples were analysed at successive dilutions, with different LAL sensitivities, to satisfy the endotoxin limits of 5 EU/ml for the concentrate and 0.25 EU/ml for the fluid for dialysis peritoneal. The results showed that the major interference on the gel-clot assay occurs in presence of acetic acid and in concentrates containing acid acetic, even the pH being adjusted between 6.5 and 7.5. However, the test, after an adequate dilution, could be validating for all samples. Chromogenic test can be used for peritoneal dialysis fluids considering a limit of 0.25 EU/ml and sample dilution of eight times, but it cannot be used for concentrates for hemodialysis without further dilution. Considering the results and that the chromogenic is a more time-consuming method, endotoxins in fluids for hemodialysis can be satisfactorily assayed by the gel-clot method.

Keywords: Hemodialysis fluids; Endotoxins; Limulus amebocyte lysate; Salt interference

1. Introduction

Endotoxins, released by gram-negative bacteria, can cause a response in humans ranging from mild fever to shock or even death. A reliable method of detecting bacterial endotoxins in phar-

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maceuticals is, therefore, of vital importance to the patients.

Dialysis patients are exposed to 400–600 l water per week to exchange organic and inorganic chemicals between dialysate and patient's vascular system, therefore, the water quality is extremely important. Tap water is a source of endotoxins that are harmless when ingested but not when they enter the blood stream. Treatments by ion-exchange or reverse-osmosis, usually provide water with good quality. However, to prepare the dialysate the chemicals are supplied in a concentrate solution that must be added to the water. For peritoneal dialysis (PD), the dialysate is supplied direct in a diluted form. If these solutions contain endotoxins, they will, in both cases, reach the patients; consequently, as important as the endotoxin water control is the endotoxin dialysate control.

According to AAMI [1], water and dialysate should be monitored for bacterial endotoxins at least monthly. The British Pharmacopeia [2] and the United States Pharmacopeia [3] establish an endotoxin limit of 0.25 EU/ml for water and dialysate, including PD. The standards recommended by AAMI for the requirements for concentrates for hemodialysis (HD) states that the supplier shall provide a statement that the solution does not exceed a limit of 5 EU/ml, when the limulus amebocyte lysate (LAL) assay is used.

The determination of endotoxins in water for hemodialysis is well-documented [4,5] but in dialysates and concentrates it is not. Due to the high saline concentration of these samples (mainly the concentrates) application of the LAL test without further investigation is not possible. HD concentrates are usually supplied in 3.6–4.0 l bottles that should be diluted in water to give 120 l dialysate, either batch before the dialysis session or on-line through a machine that mixes it with water proportionally during the session. In both cases, the investigation on the presence of endotoxins should rather be done before diluting, considering the waste of water in the first procedure and the impossibility of obtaining dialysate outside the machine in the second one.

In the last years, the rabbit pyrogen test for in vivo assay, has been substituted by an alternative in vitro test, introduced by Cooper et al. [6] and Yin et al. [7]: the limulus amebocyte lysate (LAL) test.

The LAL is a test utilising lysate of the blood cells of the horseshoe crab, which enzymatically interacts with endotoxins. The LAL test can by applied in three different ways. The gel-clot is a semi-quantitative test where, the presence of endotoxins is observed by the formation of a solid gel; whereas the chromogenic and turbidimetric are quantitative tests and measure the colour or turbidity developed by a reagent in presence of endotoxins. Currently, the LAL test is widely used to assay endotoxins in many different samples [8–11].

Most samples can be tested by any of the methods. However, for some samples, one method is preferable to the others. The gel-clot is often the best method due to its straightforward to perform and ease to interpret the results, however, if interference cannot be overcome other method should be tested. Interference occurs as inhibition or enhance of results and it is usually resolved by an adequate dilution of the sample [12–14].

Divalent cations as calcium and magnesium play an important role on the dispersion and reactivity of endotoxins. Because divalent cations neutralise the endotoxin negative charge, their concentration must be well adjusted to guarantee neither enhance nor inhibition of the LAL test [12]. In this report, the interference of each chemical (cations and anions) that constitutes HD concentrates and PD dialysate on the LAL endotoxin determination was investigate, as well as the necessary dilution to validate the test for these samples.

2. Experimental

2.1. Instrumentation and apparatus

A Perkin Elmer (Überlingen, Germany) Lambda 16 spectrophotometer, a Sonorex Super RK510 H water-bath (Germany), pyrogen-free sterile pipette tips (Oxford, USA), 10×75 mm^2 flint borosilicate glass tubes (Pyrotell, USA), a

Table 1
Concentration and pH (before adjustment) of the solutions used for individual tests

Substance	Concentration (g/l)	pH
NaCl	296.50	5.3
KCl	7.87	6.5
$CaCl_2$	10.28	7.5
$MgCl_2$	8.54	6.3
Sodium acetate	214.22	9.3
Sodium lactate	176.45	12.0
Sodium bicarbonate	84.00	8.6
Glucose	75.60	6.1
Acetic acid	20.99	2.5

Heraeus MR 170 E (Switzerland) muffle oven, a Digimed–DM 20 (Brazil) pH meter were used.

2.2. Reagents

2.2.1. Standard endotoxin

Lyophilised preparations of endotoxin of *Escherichia coli* were used throughout the experiments. The Control Standard Endotoxin (CSE) supplied by Pyrotell (USA) contained 1000 EU/ml and supplied by Endosafe (USA) 32.5, 30.0 and 20 EU/ml. The CSE were reconstituted with endotoxin-free water according to manufacturer's instruction. The reconstituted endotoxin standards were also used for spiking samples in the inhibition/enhancement tests of interfering effect.

2.2.2. LAL reagents

Three commercially available LAL reagents were used. Gel-clot with labelled sensitivities of 0.25, 0.125 and 0.03 EU/ml from Pyrotell and Endosafe and the chromogenic (Pyrochrome®) from Pyrotell. The reagents, supplied in lyophilised form, were reconstituted respectively with 5.0, 5.2 and 3.2 ml endotoxin-free water according to manufacturer's instruction.

2.2.3. Samples

The substances used to prepare endotoxin-free samples were NaCl, KCl, $CaCl_2{\cdot}2H_2O$, $MgCl_2{\cdot}6H_2O$, CH_3COONa, $NaHCO_3$, CH_3COOH, sodium lactate and glucose (all Merck, Germany). An individual solution of each substance was prepared at the concentration of HD concentrates, moreover, solutions with the same composition of the PD and concentrates containing all components were prepared. The concentration of these solutions are presented at Tables 1 and 2. To set the pH of the samples between 6.5 and 7.5, solutions of NaOH and HCl either 1.0 or 0.1 mol/l were used.

All glassware used for tests and preparation of solutions were rolled up with aluminium paper and heated at 250 °C for 30 min, or at 190 °C for 3 h.

Table 2
Composition of solutions prepared to have the same concentration of PD and concentrates for hemodialysis

Substance	Concentration (g/l)					
	PD		Concentrate[a]			
	1	2	1	2	3	4
NaCl	5.67	5.786	296.50	204.66	212.8	245.45
KCl	–	–	7.87	7.87	–	4.47
$CaCl_2{\cdot}H_2O$	0.257	0.257	10.28	10.28	9.1	8.82
$MgCl_2{\cdot}6H_2O$	0.152	0.1017	8.54	8.54	3.96	6.10
Glucose	42.5	15	75.60	62.97	31.7	40.0
Sodium acetate	–	–	214.22	–	–	–
Sodium lactate	3.92	3.924	–	176.45	–	
Sodium bicarbonate	–	–	–	–	84.0	
Acetic acid	–	–	–	–		12.01

[a] 1: acetate, 2: lactate, 3: bicarbonate, 4: acetic acid.

2.3. Preliminary procedures

After reconstitution, the CSE were diluted to make appropriate four serial two-fold interval dilutions according to the sensitivity of the LAL reagent (0.25, 0.125 and 0.03 EU/ml). The sensitivity test was performed in duplicate including a negative control.

The maximum valid dilution (MVD), maximum dilution that allows detection of the endotoxin limit, was calculated based on the limits of 0.25 EU/ml for PD and 5 EU/ml for HD concentrate. Considering the LAL reagent sensitivities of 0.25, 0.125 and 0.03 EU/ml, the MVD are for PD, undiluted, $1+1$ and $1+7$, respectively, and for HD concentrate, $1+15$, $1+31$ and $1+63$, respectively. According to this, two parallel series of samples, spiked and unspiked with the endotoxin, were performed using four two-fold serial dilution.

2.4. Standard procedure for LAL gel-clot test

The CSE dilutions were made from the standard by successive dilutions until the desired concentration (lower concentration: 0.0078 EU/ml).

Before the test, the pH of the samples were measured. Samples outside the acceptable pH range for the LAL test, had their pH adjusted with solutions of NaOH or HCl. In order to avoid excessive dilution, it was added from 30 to 300 μl of these solutions to 5 or 10 ml of sample solutions.

One hundred microlitres of standards and product samples were mixed with 100 μl LAL reagent and put into the water-bath at 37 ± 0.5 °C for 60 ± 2 min. After that, the tubes were inverted to observe the gel formation.

2.5. Standard procedure for LAL chromogenic test

The following CSE dilutions were made: 0.50, 0.25, 0.125, 0.06 and 0.03 EU/ml in order to obtain the analytical curve. Different sample volumes and incubation ratios were tested. The same volume of sample (100 or 150 μl) and LAL colour reagent were mixed and put into the water-bath at 37 ± 0.5 °C for 20 ± 2 or 30 ± 2 min. After that, 700 μl of 14.3% (v/v) acetic acid solution was added to the tube to stop the reaction. The absorbance of the solution was read at 405.0 nm. The chromogenic test was carried out with solutions of NaCl, KCl, $NaHCO_3$, CH_3COOH, and the mixtures of concentrates for hemodialysis. Samples were diluted $1+1$ and $1+7$ to be assayed.

2.6. Application to commercial hemodialysis fluids

Solutions for CAPD Dianeal 137 (Baxter) and Peritosteril (Fresenius) and concentrates for hemodialysis with lactate (B.Braun), with acetate (Salbego), with bicarbonate (Salbego) and with acetic acid (RT), were assayed for endotoxins by the gel-clot and by the chromogenic methods, utilising the necessary dilution, determined with the standards (Table 5) and following the procedures above described.

3. Results and discussion

The sensitivity of the LAL reagents was confirmed, according to the suppliers, with endotoxin spikes adequate for the investigated sensitivity.

The results of the gel-clot test with different sensitivities for all substances with the dilutions done to avoid the inhibition are in Table 3. In spite of calcium and magnesium are related as causing the major interference in the LAL test, in this assay they did not interfere, probably due to their low concentration. No inhibition was also observed in presence of glucose, sodium acetate and sodium lactate However, NaCl and KCl inhibited the test. Solutions of bicarbonate and acetic acid had to be already diluted before the test due to pH adjustment. They were diluted $1+1$ by addition of HCl and NaOH, respectively. Even being diluted these solutions needed a further dilution to overcome inhibition, bicarbonate at least $1+3$ and acetic acid $1+15$. The results were similar for both LAL reagents (Endosafe and Pyrotell), and endotoxin levels below 5 EU/ml, as recommended by AAMI, could be detected.

Table 3
Dilutions to avoid inhibition in the gel-clot method for each substance, with respect to the investigated sensitivities

Substance	Sensitivity (EU/ml)					
	LAL endosafe			LAL pyrotell		
	0.25	0.125	0.03	0.25	0.125	0.03
NaCl	1+7	1+7	1+7	1+7	1+7	1+7
KCl	1+1	1+1	1+1	1+1	1+1	1+1
$CaCl_2$	0	1+1	1+7	0	1+1	1+7
$MgCl_2$	0	1+1	1+7	0	1+1	1+7
Sodium acetate	0	1+1	1+7	0	1+1	1+7
Sodium lactate	0	1+1	1+7	0	1+1	1+7
Sodium bicarbonate	1+3	1+7	1+7	1+3	1+7	1+7
Glucose	0	1+1	1+7	0	1+1	1+7
Acetic acid	1+15	1+31	1+63	1+15	1+63	1+63

Table 4
Dilutions to avoid inhibition of the gel-clot method for different types of concentrates for hemodialysis and for PD

Concentrate	Sensitivity (EU/ml)					
	LAL endosafe			LAL pyrotell		
	0.25	0.125	0.03	0.25	0.125	0.03
Acetate	1+7	1+7	1+7	1+7	1+7	1+7
Lactate	1+7	1+7	1+7	1+7	1+7	1+15
Bicarbonate	1+7	1+7	1+7	1+7	1+7	1+7
Acetic acid	1+31	1+63	1+63	1+31	1+63	1+63
PD 1	0	1+1	1+1	0	1+1	1+1
PD 2	0	1+1	1+1	0	1+1	1+1

The analysis of the salt mixtures (concentrates) showed results expected from the results of the individual components. Concentrates with acetic acid and bicarbonate had to be diluted almost in the same ratio of the individual component (acetic acid or bicarbonate) to avoid the inhibition. Therefore, synergistic effect was not observed. The dilutions and endotoxin limits found for these samples are in Table 4.

3.1. Chromogenic method

The samples selected to be assayed by the chromogenic method were the ones that showed the higher interference with the gel-clot method. The samples were individual solutions of NaCl, KCl, acetic acid and sodium bicarbonate, besides the mixtures that correspond to the PD fluids and concentrates. Samples were spiked with the half of the endotoxin limit for PD fluids, 0.125 EU/ml. Firstly, a small dilution of 1 + 1 was tested, with sample volume of 100 μl and 20 and 30 min incubation (analytical curves 1 and 2). Only in NaCl and KCl solutions the added endotoxin was recovered (Table 5), but the absorbance values showed poor reproducibility. The following attempts were sample volume of 150 μl and incubation times of 20 and 25 min (curves 3 and 4, respectively). A bigger dilution was also tested, 1 + 7, and the results are those obtained with the curve 4. The increase of the sample volume (150 μl) with keeping the same dilution factor of 1 + 1

Table 5
Results of the chromogenic test for samples that needed high dilution to be assayed by the gel-clot

Sample	Sample dilution	Endotoxin found (EU/ml)	Recovery (%)	Curve[a]
NaCl	1+1	0.155	124	1
	1+1	0.090	72	2
	1+1	0.115	92	3
	1+7	0.147	117	4
KCl	1+1	0.153	122	1
	1+1	0.116	93	2
	1+1	0.108	87	3
	1+7	0.102	82	4
$NaHCO_3$	1+1	n.f.	–	1
	1+1	n.f.	–	2
	1+1	n.f.	–	3
	1+7	0.086	69	4
Acetic acid	1+1	n.f.	–	1
	1+1	n.f.	–	2
	1+1	n.f.	–	3
	1+7	0.024	19	4
Concentrated acetate	1+1	n.f.	–	1
	1+1	n.f.	–	2
	1+1	n.f.	–	3
	1+7	0.030	24	4
Concentrated lactate	1+1	n.f.	–	1
	1+1	n.f.	–	2
	1+1	n.f.	–	3
	1+1	0.078	63	4
Concentrated bicarbonate	1+1	n.f.	–	1
	1+1	n.f.	–	2
	1+1	n.f.	–	3
	1+1	0.051	41	4
Concentrated acetic acid	1+1	n.f.	–	1
	1+1	n.f.	–	2
	1+1	n.f.	–	3
	1+7	0.027	21	4
Solution CP 1	1+1	0.021	17	1
	1+1	0.026	21	2
	1+1	0.023	18	3
	1+7	0.118	95	4
Solution CP 2	1+1	0.024	19	1
	1+1	0.030	24	2
	1+1	0.028	22	3
	1+7	0.121	97	4

Spike: 0.125 EU/ml. Conditions: curve 1, 100 μl sample, 20 min incubation; curve 2, 100 μl sample, 30 min incubation; curve 3, 150 μl sample, 20 min incubation; curve 4, 150 μl sample, 25 min incubation.

[a] Regression functions: Curve 1, $y = 0.131x + 0.009$, $r = 0.9988$; Curve 2, $y = 0.227x–0.020$, $r = 0.9997$; Curve 3, $y = 0.426x–0.016$, $r = 0.9883$; Curve 4, $y = 0.840x + 0.002$, $r = 0.9870$.

Table 6
Analysis of commercial concentrates for hemodialysis and solutions for PD

Sample	Gel-clot			Chromogenic		
	Dilution	Spike (EU/ml)	Result	Dilution	Spike (EU/ml)	Found (EU/ml)
Concentrated acetate	1+15	–	–			
	1+15	5	+			
Concentrated lactate	1+31	–	–			
	1+31	5	+			
Concentrated acetic acid	1+127	–	–			
	1+127	5	+			
Solution PD 1	1+3	–	–	1+7	–	–
	1+3	0.25	+	1+7	0.25	0.24
Solution PD 2	1+3	–	–	1+7	–	–
	1+3	0.25	+	1+7	0.25	0.22

Results of gel-clot and chromogenic tests. Gel-clot from Pyrotell, sensitivity 0.03 EU/ml.

showed no better results. Only with a dilution factor of $1+7$ good results for PD fluids were obtained, however, for concentrate mixtures, the recoveries were still too low.

Samples that needed a very high dilution for the gel-clot and could be better assayed by the chromogenic method, showed, however, with this method, very poor results. The chromogenic reaction is very time-sensitive and for a large number of samples, it is difficult to reproduce the reaction conditions without automatic systems for mixing the reagents and stopping the reaction. It can be seen that the results are not reliable for samples assayed without high dilution. With reliable results, even being necessary a small dilution, it could be worth the use of the chromogenic method for these samples instead of promoting numerous dilutions steps necessary for the gel-clot.

3.2. Analysis of commercial samples

Six different samples were assayed by the gel-clot and chromogenic methods. They were assayed in triplicate, three samples of the same lot. Necessary dilutions and results are summarised in Table 6. All samples showed negative results, i.e., <5 EU/ml for the concentrates and 0.25 EU/ml for PD solutions.

4. Conclusion

Enhancement and inhibition of the LAL assay that result in differences in endotoxin levels at different sample dilutions might contribute to differences of reported endotoxin levels. The gel-clot assay done with reagents from different suppliers did not show the same results, but these differences do not occur at the limit to be reached.

Tests with each substance that compose the hemodialysis fluids showed that they have different influences on the result of the gel-clot assay. The stronger interference was observed by acetic acid, even being the pH adjusted adequately. Following acetic acid, sodium bicarbonate, KCl and NaCl also inhibited the reaction. Tests with mixtures also showed interference and, as expected, the major interference occurred in solutions containing acetic acid. Concentrates with acetic acid needed high dilution to be assayed, and the proposed limit of 5 EU/ml (AAMI) was only possible to reach using the reagent with sensitivity 0.03 EU/ml and diluting the sample 64 folds, dilution that does not exceed the MVD. For the other concentrates types and solutions with PD composition, lower dilutions could be used for practically all reagent sensitivities and the limit of 5 EU/ml for concentrates and 0.25 EU/ml for PD fluids could be reached. No synergistic effect was observed with the mixture of the components.

The chromogenic method could be used for PD fluids. Spiked with 0.125 EU/ml, at a dilution level of 1 + 7, they showed good recoveries.

Because of the major time and involvement of the chromogenic method and the good results of the gel-clot, the latter can be used satisfactorily for the endotoxin assay in hemodialysis fluids.

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References

[1] AAMI, Association for the Advancement for Medical Instrumentation, Hemodialysis systems, ANSI/AAMI–RD5 (1992).
[2] British Pharmacopeia, International Edition, Crown Copyright, p. 1498 (1994).
[3] United States Pharmacopoeia, USP 23, Rand McNally, p. 1184 (1995).
[4] N. Ismail, B.N. Becker, R.M. Hakim, Am. J. Nephrol. 16 (1996) 60–72.
[5] E. Klein, T. Pass, G.B. Harding, et al., Artif. Organs. 14 (1990) 85–94.
[6] J. Cooper, J. Levin, H.N. Wagner, J. Lab. Clin. Med. 78 (1971) 138–148.
[7] E.T. Yin, C. Galanos, S. Kinsky, R.A. Bradshaw, et al., Biochem. Biophys. Acta 261 (1972) 284.
[8] A. Yamamoto, M. Ochiai, H. Fujiwara, S. Asakawa, et al., Biologicals 28 (2000) 155–167.
[9] S. Zijlstra, P. Gerken, C. Rechin, R. Wortmann, et al., Appl. Radiat. Isot. 48 (1997) 51–54.
[10] L. Moesby, E.W. Hansen, J.D. Christensen, J. Clin. Pharm. Ther. 25 (2000) 283–289.
[11] G.N. Keller, R.N. Berzofsky, R. Burt, J. Stefka, et al., Cytotherapy 1 (1999) 423–428.
[12] J.F. Copper, J. Parenter. Sci. Technol. 44 (1990) 13–15.
[13] LAL Update: Volume 14, No 4, Wood Hole. Massachusetts: Associates of Cape Cod (1996).
[14] LAL Update: Volume 15, No 1, Wood Hole. Massachusetts: Associates of Cape Cod (1997).