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Specific assay for endotoxin using immobilized histidine, *Limulus* amoebocyte lysate and a chromogenic substrate

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ABSTRACT

The *Limulus* amoebocyte lysate (LAL) test is inhibited or enhanced by many substances. In order to overcome this problem, a specific endotoxin assay method using a membrane filter unit, a chromogenic LAL reagent, and immobilized histidine (which is a specific adsorbent for endotoxins) was developed. Endotoxins are quantitatively adsorbed on immobilized histidine. The adsorbed endotoxins are separated from LAL-inhibiting or -enhancing substances by the membrane filter unit, and their activities are directly assayed with the LAL reagent in a filter cup without any inhibition or enhancement. The reproducibility and the accuracy of this method are high. This new endotoxin assay method using immobilized histidine can be used for the determination of endotoxins in a solution containing LAL-inhibiting or -enhancing substances such as amino acids and antibiotics, as an alternative to the more common gel-clot technique.

INTRODUCTION

For several years, we have been studying the preparation of specific adsorbents for bacterial endotoxin [also called lipopolysaccharides (LPS) and pyrogen] and have developed immobilized histidine [1–4]. Immobilized histidine is produced by the covalent binding of histidine on agarose through hexamethylenediamine. This adsorbent has a high affinity and selectivity for endotoxin, and can be used for the removal of endotoxin from various solutions. We have been continuing to study the application of immobilized histidine.

It is necessary that pharmaceutical products intended for parenteral administration are tested for pyrogenic contamination. For the determination of the presence of endotoxins, the *Limulus* amoebocyte lysate (LAL) test is widely used because of its advantages such as low cost, high sensitivity, short operation time, good quantification and high reproducibility. However, the LAL test has some shortcomings. The principle of the LAL test is based on the endotoxin-induced coagulation reaction [5]. Unfortunately, the β-1,3-D-glucan-induced cascade also occurs, and this cascade causes a false-positive result in the LAL test. Moreover, the test is inhibited or enhanced by many substances such as antibiotics, hormones, heavy metals, amino acids, alkaloids, carbohydrates, plasma proteins, enzymes and electrolytes in the sample solution [6].

Many attempts have been made to overcome these problems. A new endotoxin-specific test, from which the β-1,3-D-glucan-sensitive factor G is removed, has been developed by Obayashi *et al.* [7]. Also, a number of possibilities such as dilution, heating, dialysis, ultrafiltration and the addition of detergents to eliminate disturbing factors have been described [8]. These methods are effective under some conditions, but they do not fully satisfy the basic requirement for elimination of interference, especially when endotoxin contamination is at very low levels. For example, in the dilution method, the sensitivity decreases because the endotoxin is also diluted. The ultrafiltration method cannot be applied to high-molecular-mass interfering substances because the pore size of membrane is small, and the

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endotoxin might be adsorbed on the ultrafiltration membrane.

In order to overcome these problems, we have attempted to develop new specific assay methods for endotoxins using immobilized histidine, and have reported one method using an ultrafiltration unit, a fluorimetric LAL reagent and immobilized histidine [9]. Immobilized histidine can adsorb endotoxins in solutions containing LAL-inhibiting or -enhancing substances. Hence it is possible to use the adsorbent as a tool for separating endotoxins from LAL-interfering substances. After separation, the activity of the endotoxins adsorbed on immobilized histidine can then be directly assayed by the LAL test without any inhibition or enhancement. This procedure had high reproducibility and accuracy and was advantageous for the determination of endotoxins in a solution containing LAL-inhibiting or -enhancing substances over the more common gel-clot technique.

However, this method still had some shortcomings: the operation time was long, the procedure was complicated, the chromogenic technique was unusable because the ultrafiltration membrane adsorbed substrate and high-molecular-mass substances could not be applied because of the small pore size of the membrane. In order to overcome these shortcomings, we have further improved this method by using a membrane filter unit and a chromogenic substrate.

In this paper, we describe the detailed conditions of endotoxin adsorption on immobilized histidine and the LAL reaction of the adsorbed endotoxin, the reproducibility and accuracy of the improved method and the assay of endotoxins in various substances by the improved method.

EXPERIMENTAL

Materials

LPS (*Escherichia coli* 0111:B4, *Salmonella enteritidis* and *Shigella flexneri*) was purchased from Difco Labs. (Detroit, MI, USA), LPS [*Salmonella minnesota* R5 (Rc) and *S. minnesota* R595 (Re)] from List Biological Labs. (Campbell, CA, USA) and LPS (*E. coli* UKT-B), US standard endotoxin (lot EC-5), *Limulus* amoebocyte lysate HS II (manufactured by Associates of Cape Cod) and Curdlan (β-1,3-D-glucan) from Wako (Osaka, Japan). The Toxicolor test was purchased from Seikagaku Kogyo (Tokyo, Japan), QCL-1000 (chromogenic LAL reagent) from Whittaker Bioproducts (Walkersville, MD, USA) and immobilized histidine (Pyro-Sep) from Tanabe Seiyaku (Osaka, Japan). Ultrafree CL-GV Retention [a unit composed of a filter cup with a membrane filter (pore size 0.22 μm) and a tube] was obtained from Nihon Millipore (Tokyo, Japan), Immersible CX-10 ultrafiltration units from Millipore (Bedford, MA, USA) and sterile test-tubes (75 × 12 mm I.D.) from Iwaki Glass (Tokyo, Japan). Crystalline penicillin G potassium was supplied by Meiji Seika Kaisha (Tokyo, Japan) and pyrogen-free water, Amizet (amino acid infusion), L-phenylalanine, L-methionine and L-cysteine hydrochlorides monohydrate by Tanabe Seiyaku (Osaka, Japan). All other chemicals were of analytical-reagent grade.

Buffer

Sodium acetate buffers (pH 4.0 and 5.5), sodium phosphate buffer (pH 7.0) and barbital buffer (pH 8.5) were used. The contaminating pyrogen in these buffers was removed by ultrafiltration with Immersible CX-10.

Endotoxin adsorption on immobilized histidine for selection of optimum adsorption conditions

In a sterile test-tube, 45–270 mg of moist immobilized histidine gel (the dry weight per gram of moist immobilized histidine gel is equivalent to 60 mg), 50 or 100 μl of LPS solution [2–500 EU (endotoxin units)/ml] and water, 25 m*M* sodium chloride solution or buffer solution were placed (total volume 0.5, 1, 2 or 3 ml) and the suspension was stirred at 50 rpm with an end-to-end mixer at 4–40°C for 2.5–60 min. After being stirred, the tube was centrifuged (2300 *g*, 5 min) and the concentration of endotoxin in the supernatant was measured by the chromogenic technique [10] using the Toxicolor test.

Depyrogenation of filter cup

A filter cup was soaked in 5% hydrogen peroxide solution and then heated at 70°C for 3 h. After heating, the filter cup was washed thoroughly with pyrogen-free water and dried at 60°C.

Preparation of pyrogen-free sample solution

Pyrogen was removed from various solutions as follows. In 20 ml of a sample solution, 0.3 g of moist immobilized histidine gel was suspended and the suspension was stirred at room temperature (25°C) for 4 h. After being stirred, the adsorbent was separated by centrifugation (2300 *g*, 5 min).

Assay of endotoxin by the immobilized histidine method

Adsorption. In a filter cup, 300 μl of 30% [w/v (wet weight)] immobilized histidine suspension, 100–1600 μl of buffer solution and 100 μl of sample solution were placed, and the suspension was shaken at 25°C for 15 min using a Micromixer MT (Taitec, Tokyo, Japan). After being shaken, the filter cup was drained by suction for 2 min using a Millititer vacuum holder (Millipore) and the filtrate was discarded. To the filter cup, 1 ml of 20 m*M* sodium chloride solution was added and shaken for 5 min using a Micromixer MT. After being shaken, the filter cup was drained by suction for 5 min.

LAL test. One vial of substrate (QCL-1000) was dissolved in 6.5 ml of water. The contaminating pyrogen in 0.4 *M* Tris–HCl buffer (pH 8.0) containing 0.04 *M* magnesium chloride was removed by ultrafiltration with Immersible CX-10. One vial of LAL (QCL-1000) was dissolved in 1.4 ml of water or the diluted form of the above buffer. The filter cup containing the endotoxins adsorbed on the adsorbent was preincubated at 37°C for 10 min. After preincubation, 100 μl of LAL solution and 200 μl of substrate solution were added to the filter cup and the suspension was incubated at 37°C for 20–40 min using a QA Model DP-110 thermomixer (Erma, Tokyo, Japan) with shaking. After incubation, the reaction was stopped by adding 200 μl of 25% acetic acid and the mixture was filtered with suction for 2 min. The absorbance of the filtrate was measured at 405 nm.

Measurement of gel clotting activity

The sample solution was diluted with pyrogen-free water. One vial of *Limulus* amoebocyte lysate HS II was dissolved in 5 ml of pyrogen-free water. A 100-μl volume of sample solution and 100 μl of *Limulus* amoebocyte lysate HS II solution were incubated at 37°C for 60 min. After the incubation, the gel clotting in the tube was examined.

RESULTS

Optimum conditions for adsorption

For a quantitative assay of endotoxin using immobilized histidine, it is necessary that endotoxins be adsorbed on immobilized histidine completely, and that the endotoxins adsorbed on immobilized histidine show sufficient activity.

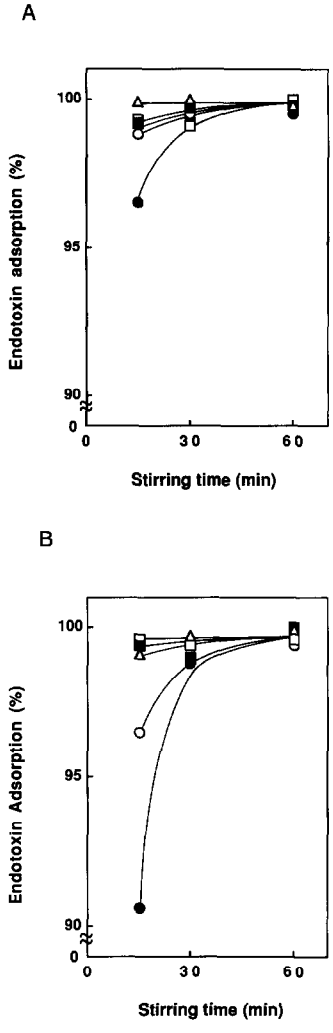

Fig. 1. Effect of the amount of adsorbent and adsorption time on endotoxin adsorption. Endotoxin adsorption was carried out as described in the text using 0.2 EU of EC-5, (●) 45, (○) 90, (□) 135, (■) 180 or (△) 270 mg of moist immobilized histidine, with a total volume of 3 ml in (A) water or (B) 25 m*M* sodium chloride solution. Endotoxin concentration in the supernatant was measured as described in the text.

First, the optimum conditions for endotoxin adsorption on immobilized histidine were investigated. The adsorption capacity of immobilized histidine is 0.74 mg of LPS (*E. coli* 0128:B12) or 0.31 mg of LPS (*E. coli* UKT-B) per gram of moist immobilized histidine gel. The dissociation constants of immobilized histidine for LPS (*E. coli* 0128:B12 and *E. coli* UKT-B) are $1.57 \cdot 10^{-9}$ and $7.3 \cdot 10^{-13}$ *M*, respectively, when the molecular mass of LPS is taken as 10^6 [3,4]. The effects of the amount of adsorbent and adsorption time on endotoxin adsorption are shown in Fig. 1. The endotoxin adsorption increased with increase in the amount of adsorbent and the stirring time. The adsorption of endotoxin in water was more rapid than that in 25 m*M* sodium chloride solution. In both instances, however, using more than 135 mg of moist adsorbent per 3 ml, the adsorption of endotoxin was higher than 99% within 15 min.

The effects of pH, ionic strength (μ) and endotoxin concentration on endotoxin adsorption are shown in Table I. In the sodium chloride solution, the adsorptions of endotoxin were relatively low, but in buffer of pH 4–7 and at ionic strength <0.05, the adsorptions of endotoxin were higher than 99% at every endotoxin concentration.

The effect of temperature on endotoxin adsorption is shown in Fig. 2. At ionic strength <0.05, at every temperature, endotoxin was well adsorbed on the adsorbent. However, at pH 7 and ionic strength 0.1, the adsorption of endotoxin was a function of temperature, increasing with increase in the temperature of adsorption. The adsorption of endotoxin was higher than 98% at 40°C.

The adsorptions of various kinds of endotoxins on the adsorbent are shown in Table II. At a reaction volume of 2 ml, the adsorption of endotoxin from *Salmonella enteritidis* was low. At a reaction volume of 1 ml, however, the adsorption of every endotoxin tested was higher than 97%.

From these results, at adsorption conditions of 90 mg of moist immobilized histidine gel, pH 4–7, ionic strength <0.05, reaction volume 1 ml, and adsorption time 15 min, endotoxins were adsorbed quantitatively on immobilized histidine.

Using these conditions for adsorption, we then investigated the LAL reaction step.

Optimum conditions for LAL test

LAL reagent was added to endotoxins adsorbed on immobilized histidine. Therefore, it is necessary that endotoxins adsorbed on immobilized histidine show sufficient activity.

The effect of the Tris buffer concentration in LAL on the LAL activation by the endotoxin adsorbed on immobilized histidine is shown in Table III. A chromogenic LAL reagent, QCL-1000, was

TABLE I

EFFECT OF pH, IONIC STRENGTH AND ENDOTOXIN CONCENTRATION ON ENDOTOXIN ADSORPTION

Endotoxin adsorption was carried out as described in the text using 0.2–50 EU of EC-5, 90 mg of the adsorbent, a total volume of 2 ml and a stirring time of 15 min. Endotoxin concentration in the supernatant was measured as described in the text.

Adsorption conditions	Ionic strength (μ)	Endotoxin adsorption (%)			
		EC-5 added (EU)			
		0.2	0.5	5	50
Water	0	–	99.7	99.9	99.9
NaCl	0.01	–	99.7	99.9	99.9
	0.02	–	99.8	99.9	99.9
	0.025	99.6	–	–	–
	0.05	–	86.0	85.8	75.8
	0.1	–	56.9	14.7	–
	0.2	–	65.0	7.8	–
pH 4.0	0.01	–	99.6	99.9	99.9
	0.02	–	99.6	99.9	–
	0.05	–	99.6	100.0	99.8
	0.1	–	99.8	99.9	99.8
	0.15	–	63.6	97.3	99.0
	0.2	–	66.4	96.6	98.7
pH 5.5	0.01	–	99.4	99.9	99.8
	0.02	–	99.9	99.9	–
	0.05	–	99.3	99.9	99.8
	0.1	–	99.2	99.8	99.8
	0.15	–	94.9	99.2	98.2
	0.2	–	92.4	81.7	56.9
pH 7.0	0.01	–	99.6	99.8	99.8
	0.02	–	99.5	99.9	–
	0.05	–	99.8	99.8	99.8
	0.1	–	96.9	94.3	95.9
	0.15	–	87.3	67.2	48.0
	0.2	–	75.7	29.3	–
pH 8.5	0.01	–	99.9	99.8	99.8
	0.02	–	99.9	99.8	99.8
	0.05	–	96.9	90.2	97.0
	0.1	–	66.1	9.1	–
	0.15	–	22.4	1.8	–
	0.2	–	42.8	–	–

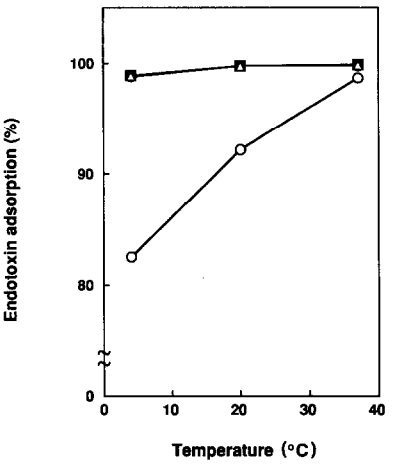

Fig. 2. Effect of temperature on endotoxin adsorption. Endotoxin adsorption was carried out as described in the text using 5 EU of EC-5, 90 mg of the adsorbent, a total volume of 2 ml in buffer (pH 7.0) and a stirring time of 15 min at various temperatures. Endotoxin concentration in the supernatant was measured as described in the text. Ionic strength (μ): □ = 0.01; ● = 0.02; △ = 0.05; ○ = 0.1.

TABLE II

ADSORPTION OF VARIOUS KINDS OF ENDOTOXINS ON IMMOBILIZED HISTIDINE

Endotoxin adsorption was carried out as described in the text using about 0.5 EU of endotoxin, 90 mg of the adsorbent, a total volume of 1 or 2 ml, phosphate buffer (pH 6, μ = 0.04) and a stirring time of 15 min. Endotoxin concentration in the supernatant was measured as described in the text.

Endotoxin	Endotoxin adsorption (%)	
	Adsorption volume 1 ml	Adsorption volume 2 ml
EC-5 (*E. coli* 0113)	100.0	99.4
E. coli UKT-B	100.0	99.3
E. coli 0111: B4	99.7	97.5
Salmonella enteritidis	97.8	93.5
Shigella flexneri	98.8	99.2
Lipid A from *E. coli* J5 mutant	100.0	99.6
R5 (Rc) from *Salmonella minnesota*	100.0	99.8
R595 (Re) from *Salmonella minnesota*	100.0	99.9

TABLE III

EFFECT OF TRIS BUFFER CONCENTRATION IN LAL ON LAL ACTIVATION BY THE ENDOTOXIN ADSORBED ON IMMOBILIZED HISTIDINE

Endotoxin adsorption was carried out as described in the text using 0.1 EU of EC-5, 90 mg of the adsorbent, a total reaction volume of 1 ml of water and a stirring time of 15 min. The LAL reaction was carried out as described in the text using the LAL dissolved in various concentrations of Tris buffer and a reaction time of 30 min.

Tris buffer concentration in LAL (m*M*)	Relative activity[a]
0	100[b]
12.5	106.3
25.0	108.5
50.0	113.7
100.0	113.9
200.0	108.0
300.0	102.4

[a] The activity was taken as 100 when water was used as the dissolution buffer of LAL.

[b] The recovery of endotoxin activity was 73.2% in comparison with free endotoxin.

used as the LAL reagent. The LAL was dissolved with water or various concentrations of Tris buffer. Increasing the concentration of Tris buffer led to an increase in relative activity, with an optimum at 100 m*M* Tris buffer. Higher concentrations of Tris led to a decline in relative activity.

The effect of the adsorption conditions of endotoxin on the LAL reaction is shown in Table IV. Under all adsorption conditions tested almost the same activity was obtained.

The activity of various kinds of endotoxins is shown in Table V. The activity obtained using adsorption conditions of pH 6 and ionic strength 0.04 was compared with that obtained using 10 m*M* sodium chloride solution. Every endotoxin showed the same activity in both instances. Moreover, the adsorption temperature did not affect the LAL activity at ionic strength <0.04.

Hence it was concluded that endotoxins adsorbed on immobilized histidine showed sufficient activity.

Effect of non-specific adsorption of endotoxin on endotoxin assay

Fig. 3 and Table VI show the effect of the non-

TABLE IV

EFFECT OF ENDOTOXIN ADSORPTION CONDITIONS ON LAL REACTION

Endotoxin adsorption was carried out as described in the text using 0.1 EU of EC-5, 90 mg of the adsorbent, a total volume of 1 ml and a stirring time of 15 min. The LAL reaction was carried out as described in the text using the LAL dissolved in 0.1 *M* Tris buffer and a reaction time of 30 min.

Adsorption conditions	Relative activity[a]
Water	100
20 m*M* NaCl	104.2
pH 5.5, μ = 0.02	103.3
pH 7.0, μ = 0.02	96.2
pH 6.0, μ = 0.02	97.3
pH 6.0, μ = 0.04	100.7
pH 6.0, μ = 0.1	98.7

[a] The activity was taken as 100 when water was used for adsorption.

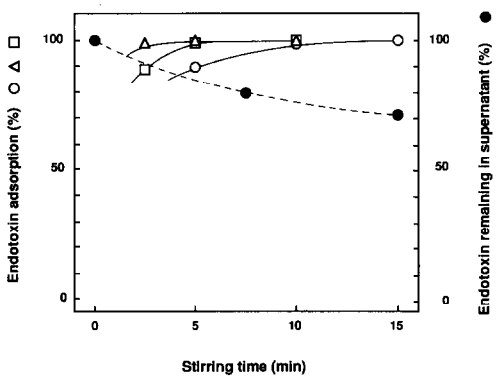

Fig. 3. Effect of the non-specific adsorption of endotoxin. Endotoxin adsorption was carried out as described in the text using 0.1 EU of EC-5, 90 mg of the adsorbent and total volume (△) 0.5, (□) 1 and (○) 2 ml in buffer (pH 6, μ = 0.04). Endotoxin concentration in the supernatant was measured as described in the text. Non-specific adsorption of endotoxin on the filter cup (●) was measured using 0.1 EU of EC-5 and a total volume of 2 ml in buffer (pH 6, μ = 0.04) without the adsorbent.

specific adsorption of endotoxin on the endotoxin assay. As shown in Fig. 3, endotoxins were non-specifically adsorbed on the filter cup, and this non-specific adsorption increased with increase in adsorption time. However, when a small adsorption volume was used, rapid adsorption of endotoxin on immobilized histidine occurred. Therefore, it is considered that the effect of non-specific adsorption was slight. Table VI shows the effect of adsorption volume on the LAL reaction. The adsorption volume did not affect the relative activity. Hence it is considered that this non-specific adsorption did not affect the LAL reaction.

Based on these results, the standard operation of the immobilized histidine method was formulated as follows. In a filter cup, 900 μl of 10% immobilized histidine suspension (pH 6, μ = 0.02) and 100 μl of sample solution were placed and the suspension was shaken at 20–40°C for 15 min using a Mi-

TABLE V

ACTIVITY OF VARIOUS KINDS OF ENDOTOXINS

Endotoxin adsorption was carried out as described in the text using about 0.1 EU of endotoxin, 90 mg of the adsorbent, a total volume of 1 ml, 10 m*M* sodium chloride solution or phosphate buffer (pH 6, μ = 0.04) and a stirring time of 15 min. The LAL reaction was carried out as described in the text using the LAL dissolved in 0.1 *M* Tris buffer and a reaction time of 30 min.

Endotoxin	Relative activity[a]
EC-5 (*E. coli* 0113)	100.7
E. coli UKT-B	102.0
E. coli 0111: B4	96.3
Shigella flexneri	98.1
R5 (Rc) from *Salmonella minnesota*	110.4
R595 (Re) from *Salmonella minnesota*	108.4

[a] The activity was taken as 100 when adsorption was carried out in 10 m*M* sodium chloride solution.

TABLE VI

EFFECT OF ADSORPTION VOLUME ON LAL REACTION

Endotoxin adsorption was carried out as described in the text using 0.1 EU of EC-5, 90 mg of the adsorbent, a total volume of 0.5, 1.0 or 2.0 ml, phosphate buffer (pH 6, μ = 0.04) and a stirring time of 15 min. The LAL reaction was carried out as described in the text using the LAL dissolved in 0.1 *M* Tris buffer and a reaction time of 30 min.

Adsorption volume (ml)	Relative activity[a]
0.5	102.8
1.0	100
2.0	99.6

[a] The activity was taken as 100 when the adsorption volume was 1.0 ml.

cromixer MT. After shaking, the filter cup was filtered for 2 min using a vacuum holder and the filtrate was discarded. For washing the adsorbent, 1 ml of 20 m*M* sodium chloride solution was added and shaken for 5 min using a Micromixer MT. After shaking, the filter cup was filtered with suction for 5 min and the filtrate was discarded. The filter cup was preincubated at 37°C for 10 min on a QA thermomixer. For LAL reaction, 300 μl of mixed solution containing both LAL reagent and substrate (QCL-1000) were added to the filter cup and the suspension was incubated at 37°C for 20–40 min using a QA thermomixer with shaking. After incubation, the reaction was stopped by adding 200 μl of 25% acetic acid solution and the mixture was filtered for 2 min. The absorbance of the filtrate was measured at 405 nm.

In practice, the sensitivity of the endotoxin assay could be changed by changing the incubation time with the LAL reagent. With an unknown sample, many dilutions of the sample would be used.

Reproducibility

The reproducibility of the immobilized histidine method is shown in Table VII. When endotoxin was not added, the mean absorbance at 405 nm, blank value (n = 6), was 0.1255, and the relative standard deviation (R.S.D.) was 2.86%. When endotoxin was added at concentrations of 0.5 and 1.0 EU/ml, the mean values of the absorbance (n = 6) were 0.5723 and 1.0523 and the R.S.D.s were 1.96 and 1.81%, respectively.

Calibration

The relationship between endotoxin concentration and absorbance at 405 nm was investigated. As shown in Fig. 4, when the time of the enzyme reaction was 20, 30 or 40 min, the calibration graphs obtained between 0 and 8, 0 and 1.6 or 0 and 0.4 EU/ml, respectively, showed good linearity.

The relationships between endotoxin concentration and absorbance using three kinds of endotoxins, *E. coli* UKT-B, EC-5 and *E. coli* 0111:B4, are shown in Fig. 5. Each regression curve showed a good linear dose–response relationship, and the regression curves were parallel. Therefore, various kinds of endotoxins can be determined by the immobilized histidine method.

TABLE VII

REPRODUCIBILITY OF THE IMMOBILIZED HISTIDINE METHOD

Endotoxin (EC-5) solution was assayed by the standard procedure (n = 6).

Parameter	Endotoxin added (EU/ml)		
	0	0.5	1.0
Absorbance (405 nm)	0.122	0.500	1.078
	0.131	0.538	0.973
	0.115	0.533	0.990
	0.111	0.533	1.051
	0.114	0.539	1.022
	0.114	0.552	1.072
Mean	0.1178	0.5325	1.0310
S.D.	0.00677	0.01586	0.03962
R.S.D. (%)	5.75	2.98	3.84

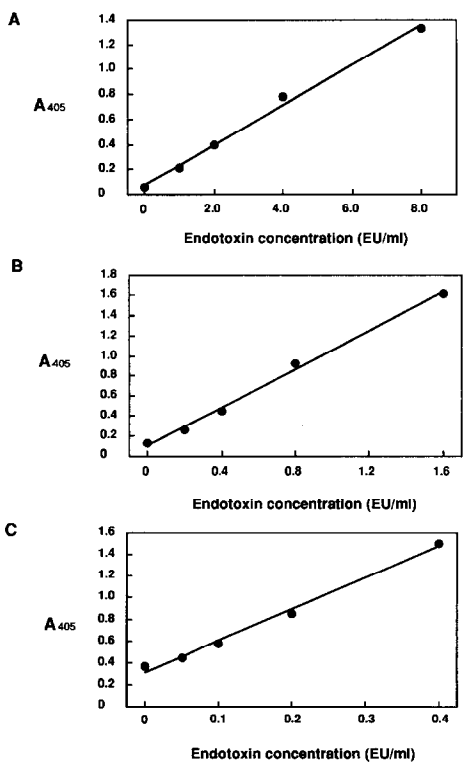

Fig. 4. Calibration graphs for the immobilized histidine method. Endotoxin (EC-5) solution was measured by the standard procedure. Time of enzyme reaction: A, 20; B, 30; C, 40 min.

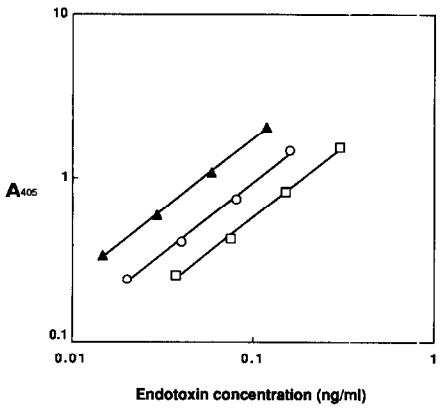

Fig. 5. Calibration graphs for various endotoxins. Endotoxin (▲ = *E. coli* UKT-B; ○ = EC-5; □ = *E. coli* 0111:B4) solution was measured by the standard procedure. The time of the enzyme reaction was 30 min.

Assays of endotoxins in various substances

Endotoxins in various substances were assayed by the immobilized histidine method. Table VIII shows a comparison of this immobilized histidine method with the gel-clot technique for endotoxin assay. In the gel-clot technique, 1.0 and 2.0 *M* sodium chloride, phenylalanine, methionine, cysteine hydrochloride monohydrate and amino acid infusion gave false-negative results because these substances inhibit the LAL procedure. On the other hand, β-1,3-D-glucan gave a false-positive result because it activates the LAL procedure. With the immobilized histidine method, the concentration of endotoxin could be accurately assayed in every instance. This indicates that the new adsorption method using immobilized histidine is a favourable assay method for endotoxins in solutions containing LAL-inhibiting or -enhancing substances.

DISCUSSION

The established LAL test has some shortcomings, such as false-negative results, in the presence of LAL-inhibiting substances, and false-positive results in the presence of β-1,3-D-glucan or other LAL-enhancing substances. We have tried to im-

TABLE VIII

ASSAYS OF ENDOTOXINS IN VARIOUS SUBSTANCES

EC-5 was added to solutions containing various substances and then the immobilized histidine method was applied or, after dilution with endotoxin-free water, the gel-clot technique was applied.

Substance	Concentration	Immobilized histidine method (EU/ml)				Gel clotting activity[a]		
		Endotoxin added (EU/ml)				Endotoxin added (EU/ml)	Dilution	Clotting
		0	0.1	0.2	0.5			
Water	–	–	–	–	–	0.2	× 6	(+)
Water	–	–	–	–	–	0.2	× 12	(−)
NaCl	0.15 *M*	<0.01	–	–	0.52	0.5	× 15	(+)
NaCl	0.5 *M*	<0.01	–	–	0.52	0.5	× 15	(+)
NaCl	1.0 *M*	<0.01	–	–	0.51	0.5	× 15	(−)[b]
NaCl	2.0 *M*	<0.01	–	–	0.42	0.5	× 15	(−)[b]
Glucose	50%	0.01	–	–	0.55	0.5	× 15	(+)
Phenylalanine	2%	<0.01	0.10	–	–	0.1	× 3	(−)[b]
Methionine	5%	<0.01	0.10	–	–	0.1	× 3	(−)[b]
Cysteine–HCl	1.5%	0.02	–	–	0.50	0.5	× 15	(−)[b]
Amino acid infusion	Original	<0.01	–	0.20	–	0.2	× 6	(−)[b]
Penicillin G potassium	10^5 U/ml	0.01	–	–	0.58	0.5	× 15	(+)
β-1,3-D-Glucan	2 ng/ml	0.06	–	–	0.55	0.5	× 25	(+)[c]

[a] Lysate sensitivity: 0.03 EU/ml.
[b] False-negative.
[c] False-positive.

prove the LAL test, and in a previous paper [9] we reported a specific assay method for endotoxin using an ultrafiltration unit, a fluorimetric LAL reagent and immobilized histidine. However, that method also had some shortcomings as described. Therefore, we have continued to try to improve the method further.

First, the conditions for adsorption of endotoxin on immobilized histidine were reinvestigated. In the previous method, an adsorption time of 1 h was needed. However, by increasing of the amount of immobilized histidine, the adsorption time could be shortened to 15 min (Fig. 1). Under this condition, in buffer of pH 4–7 and with ionic strength <0.05, the adsorption of endotoxin was higher than 99% (Table I). As regards the adsorption temperature, only at ionic strength 0.1 the adsorption increased at high temperature (Fig. 2). Moreover, various kinds of endotoxins were well adsorbed on the adsorbent.

Second, the conditions for the LAL test were reinvestigated. In the previous method, an ultrafiltration unit was used as the test tube. The ultrafiltration unit can be applied for this assay, but the filtration time is long, the chromogenic substrate is adsorbed and high-molecular-mass substances cannot be applied because the pore size of the membrane is small. In this work, we changed the test tubes from ultrafiltration units to membrane filter units. This unit has a hydrophobic membrane under the membrane filter. It is possible to retain liquids in the unit by using this double membrane and, if necessary, to remove them by suction. The pore size of this membrane filter is 0.22 μm. Therefore, the filtration time is short and it is possible to determine the endotoxin concentration in high-molecular-mass substances. Moreover, this membrane filter unit does not adsorb substrates. Therefore, the chromogenic technique can be applied. We used QCL-1000 as the chromogenic LAL reagent. As shown in Table III, when the LAL was dissolved in 100 m*M* Tris buffer, the highest recovery of endotoxin activity was observed. This might be due to the increase in the liberation of endotoxin from the adsorbent considering the characteristics of immobilized histidine [3]. In this study, the recovery of endotoxin activity was about 83% in comparison with free endotoxin. However, this recovery increased to more than 90% when the conditions of the LAL reaction were optimized. It might be difficult to obtain a complete recovery of endotoxin activity because some period is needed for the liberation of the adsorbed endotoxin from the adsorbent. However, as most kinds of endotoxins showed the same recovery, it might be possible to assay various kinds of endotoxins by the immobilized histidine method. Under these conditions, the adsorption conditions did not affect the activity of the adsorbed endotoxin (Table IV). Many kinds of endotoxins showed constant activity in spite of the adsorption conditions (Table V).

The membrane filter unit adsorbs endotoxin non-specifically. Therefore, the effect of this non-specific adsorption of endotoxin on the endotoxin assay was investigated. As a result, this non-specific adsorption did not affect the LAL reaction because of the high affinity of immobilized histidine for endotoxins (Fig. 3, Table VI).

The method reported here is simpler and more rapid than earlier versions. Previously, an end-to-end mixer for adsorption, centrifugation for separation and a water-bath for LAL reaction were used. Here, a Micromixer MT (shaking) for adsorption, filtration for separation and a QA thermomixer (block heater) were used with gains in efficiency.

This latest endotoxin assay showed good reproducibility and accuracy. Further, when endotoxins were added to solutions containing LAL-inhibiting or -enhancing substances such as amino acids and β-1,3-D-glucan and were assayed by this method, the concentration of endotoxin could be accurately determined. On the other hand, when the older gel-clot technique was used, many solutions showed false-negative or false-positive results (Table VIII).

Hence this assay method using a membrane filter unit, a chromogenic LAL reagent and immobilized histidine overcame most of the shortcomings of the previous method using an ultrafiltration unit, a fluorimetric LAL reagent and immobilized histidine, that is, the operation time is shortened, chromogenic technique can be applied and high-molecular-mass substances can be applied. By using this improved assay method, the concentration of endotoxins in solutions containing LAL-inhibiting or -enhancing substances can be assayed within 1.5 h. Further studies on the application of this immobilized histidine method are in progress.

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