

Single-Step, Chromogenic *Limulus* Amebocyte Lysate Assay for Endotoxin

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A new reagent for the chromogenic *Limulus* amebocyte lysate (LAL) assay is described. LAL was formulated for optimal performance in either an endpoint procedure or a kinetic procedure with the chromogenic substrate, buffer, and LAL components colyophilized as a single reagent. The kinetic chromogenic method required an incubating microplate reader coupled to a computer for collection and analysis of data. The kinetic method had a longer incubation time than the endpoint method and spanned a range of over 3 orders of magnitude compared with the 1-order-of-magnitude range of the endpoint assay. The kinetic method was less subject to operator error, since readings were continuous and automatic. The endpoint test was more operator intensive, requiring both addition of acetic acid to stop the reaction and transfer of the sample to the reading device. A single-step chromogenic reagent was also prepared without lyophilization by mixing reconstituted gel clot LAL with a buffer and a chromogenic substrate. The reagent prepared in this manner performed as well as the colyophilized agent.

Since the first chromogenic application of the *Limulus* amebocyte lysate (LAL) test was described in 1977 (8), numerous modifications of the technique have appeared in the literature (1-5, 8, 10, 11, 13, 16, 17). These have all been endpoint methods which involve addition of multiple reagents. The LAL enzymatic cascade is activated by endotoxin at pH 6.0 to 7.5, the amount of activated enzyme present is measured by its ability to cleave a chromogenic substrate at pH 8.0 to 9.0, and the reaction is stopped by addition of acetic acid. One method (2) used kinetics of color formation to quantify endotoxin. However, monitoring the kinetics of color development after the endotoxin activation of the LAL enzyme system offers no advantage over the other endpoint methods, since these methods have already been optimized for color formation (3).

The first true kinetic chromogenic LAL method described (17) monitored changes in absorbance over 60 min. However, this method was useful only with low endotoxin concentrations, and the researchers could demonstrate no advantage over the kinetic turbidimetric method.

All previously described endpoint chromogenic methods, as well as endpoint turbidimetric methods (15), have ranges of detection limited to little more than 1 order of magnitude. By contrast, the kinetic turbidimetric method successfully used the kinetics of endotoxin activation of the LAL enzymatic cascade (measured as turbidity) to increase the range of detection to 5 orders of magnitude (6, 7, 9, 12).

This report describes both an endpoint method and a kinetic chromogenic LAL method which use a single reagent mixture. The reagent can be prepared by colyophilization of a buffer, LAL, and a substrate (chromogenic LAL reagent) or can be mixed for immediate use with commercially available gel clot LAL, a substrate, and a buffer (gel clot-chromogenic LAL reagent). A single reagent makes possible a kinetic chromogenic method as well as a single-step endpoint (fixed incubation time) method. By eliminating multiple steps and technician error, variability in the chro-

mogenic test (16) can be greatly reduced. A detailed comparison of the kinetic and endpoint chromogenic procedures was made. The single-reagent methods were compared with a standard multiple-reagent procedure.

MATERIALS AND METHODS

Chromogenic LAL reagent. The chromogenic LAL reagent was composed of a buffer, a chromogenic substrate, and LAL. The components were processed separately and combined in a ratio of 1:1:0.75, respectively. The reagent was dispensed into 10-ml serum vials at 2.5 ml per vial and lyophilized. Vials were stoppered in vacuo and stored in the dark at 2 to 8°C until used.

Buffer preparation. An imidazole-triethanolamine buffer (pH 8.5) with added calcium and magnesium was used. Imidazole (Sigma Chemical Co., St. Louis, Mo.), triethanolamine hydrochloride (Sigma), mannitol (Sigma), anhydrous calcium chloride (Aldrich Chemical Co., Milwaukee, Wis.), and magnesium chloride hexahydrate (Sigma) were dissolved separately in distilled water and mixed to yield a solution of the following proportions: 0.05 M imidazole-triethanolamine, 8% (wt/vol) mannitol, 0.02 M CaCl₂, and 0.05 M MgCl₂. The final solution was autoclaved after it was passed through a 20,000-dalton-cutoff ultrafilter (Pyrosart SM 14571; Sartorius, Göttingen, Federal Republic of Germany) to remove endotoxin.

Substrate preparation. The chromogenic substrate CH₃OCO-D-HHT-Gly-Arg-pNA-AcOH (methoxycarbonyl-D-hexahydroxyroxyglycyl-arginine-p-nitroanilide acetate salt; Spectrozyme 200; American Diagnostica Inc., Greenwich, Conn.) was dissolved in distilled water to a final concentration of 2.2 μmol/ml. The final solution was ultrafiltered (Ultrasart D 20; Sartorius) and stored at 2 to 8°C in the dark until used.

LAL preparation. LAL, chloroform extracted as described previously (14) and containing 0.17 M NaCl, was prepared and stored at 2 to 8°C in the dark until used.

Gel clot-chromogenic LAL reagent. A single-reagent chromogenic LAL was prepared immediately before use by mixing freshly reconstituted gel clot LAL (lot no. 99-73-416;

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Associates of Cape Cod, Inc., Falmouth, Mass.), a buffer, and a substrate solution in a ratio of 1:1:1.

Additional reagents. LAL reagent water was prepared by distillation and confirmed to contain less than 0.001 endotoxin unit (EU) per ml by the kinetic turbidimetric LAL method (9). A 50% (vol/vol) mixture of glacial acetic acid in water was used as a stop solution for the endpoint assay. Reference standard endotoxin, *Escherichia coli* lot EC-5 (Office of Biologics, U.S. Food and Drug Administration), and control standard endotoxin lot 36 (Associates of Cape Cod) were reconstituted by manufacturer instructions and diluted to 1.0, 0.5, 0.25, and 0.125 EU/ml. Dilutions were prepared daily from reconstituted stock solutions (2,000 EU/ml and 100 ng/ml for reference standard endotoxin and control standard endotoxin, respectively) and stored on ice until used. Control standard endotoxin lot 36 was assigned a potency of 10 EU/ng by the method described in U.S. Food and Drug Administration guidelines (Food and Drug Administration, *Guideline on Validation of the Limulus Amebocyte Lysate Test as an End-Product Endotoxin Test for Human and Animal Parenteral Drugs, Biological Products, and Medical Devices*, December 1987, p. 1-39, U.S. Government Printing Office, Washington, D.C.).

Microplates. Flat-bottom, nonsterile polystyrene microplates were used (Linbro; Flow Laboratories, Inc., McLean, Va.).

Endpoint method with chromogenic LAL reagent. LAL reagent water (0.1 ml; negative control) was added to the first of five flint (soda lime) glass test tubes (10 by 75 mm; Fisher Scientific Co., Pittsburgh, Pa.) which had previously been depyrogenated by heating at 180°C for 4 h. Into each of the four additional tubes we added 0.1 ml of a different concentration of reference standard endotoxin or control standard endotoxin (1.0, 0.5, 0.25, or 0.125 EU/ml). A 0.1-ml volume of LAL reagent was rapidly added to all of the tubes and mixed thoroughly. The tubes were placed in a 37°C water bath or block heater and allowed to incubate for 15 to 20 min (the optimal time was determined by experimentation). Following incubation, 0.05 ml of 50% acetic acid was added to each tube to stop the reaction. At any time following the addition of acid, the solutions may be read in a spectrophotometer at 405 nm. To more accurately compare the results of the endpoint test with those of the kinetic method, 0.2 ml of each solution was added to the wells of microplates and read at 405 nm in a microplate reader (Titertek Twinreader; Flow Laboratories).

Endpoint method with multiple reagents. Endotoxin (0.05 ml) was mixed with 0.05 ml of gel clot LAL (lot no. 99-73-416; Associates of Cape Cod) in a flint glass reaction tube. After 15 min of incubation at 37°C, 0.1 ml of a 1:1 mixture of buffer-Spectrozyme (2.2 µM) was added. The tube was reincubated for 3 min, and the reaction was stopped by addition of 0.05 ml of 50% acetic acid. This solution (0.2 ml) was read in a microplate reader at 405 nm.

Kinetic method with chromogenic LAL reagent. Serial dilutions of endotoxin in the range of 10.00 to 0.03125 EU/ml were made in borosilicate reusable depyrogenated test tubes (18 by 150 mm). Samples (0.1 ml) of each dilution were dispensed into microplate wells. Chromogenic LAL was reconstituted with LAL reagent water and sonicated for 20 s with a probe sonicator (Branson Sonic Power Co., Danbury, Conn.) to clarify and uniformly disperse the suspension. The sonicator probe had been previously depyrogenated by repeated sonications with fresh LAL reagent water. Chromogenic LAL was added in 0.1-ml amounts, and the microplates were immediately and thoroughly shaken for 1 min

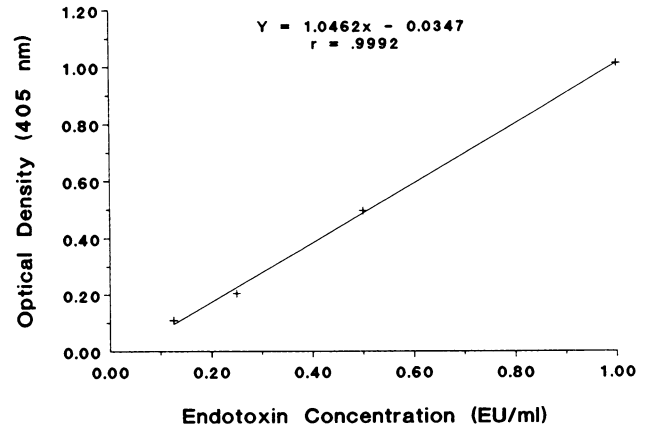


FIG. 1. Standard curve for the endpoint method using the single-step chromogenic LAL reagent. The test was incubated for 45 min at 37°C.

on the Titertek shaker. The plates were placed in the incubator-reader (Titertek Twinreader), and the reaction was allowed to proceed for 60 min. With the Twinreader Apple Program (12), the increase in optical density at 405 nm was recorded every 2 min, transmitted to an Apple IIGS computer, and stored on a disk. The time of onset (T_o) or the time at which the color reached an optical density of 100 milliabsorbance units was calculated by the computer. A standard line was plotted as the log of the T_o versus the log of the endotoxin concentration and used to calculate the endotoxin concentrations of the unknowns.

RESULTS

Endpoint method with chromogenic LAL reagent. For the endpoint method, the endotoxin and LAL reagent were incubated for 45 min. A standard curve was generated by plotting the optical density against the endotoxin concentration (Fig. 1). The parameters of the line were a slope of 1.0462, a y intercept of -0.0347, and an r value of 0.9992. A slope ranging between 0.5 and 1.5 and an r value greater than 0.98 for standards from 0.125 to 1.0 EU/ml are in accord with the guidelines of the Food and Drug Administration.

Reproducibility. The reproducibility of this LAL, both vial to vial and intravial, yielded coefficients of variation of 12% or lower. The average of 20 standard curves from 20 vials is outlined in Table 1. All tests were performed with the same endotoxin dilutions on the same day.

Ten standard curves were run with the same pooled batch of LAL to determine intravial reproducibility. The coeffi-

TABLE 1. Reproducibility of chromogenic LAL reagent with the endpoint method

Endotoxin concn (EU/ml)	Mean \pm SD endotoxin concn (coefficient of variation [%]) measured:	
	Vial to vial ^a	Intravial ^b
0.125	0.110 \pm 0.012 (10.91)	0.071 \pm 0.005 (7.04)
0.25	0.205 \pm 0.025 (12.20)	0.185 \pm 0.009 (4.87)
0.5	0.497 \pm 0.043 (8.65)	0.388 \pm 0.026 (6.7)
1.0	1.011 \pm 0.070 (6.92)	0.860 \pm 0.042 (4.88)

^a Twenty vials were used, and the slope, y intercept, and r value were 1.048, -0.03, and 0.998, respectively.

^b Ten vials were used, and the slope, y intercept, and r value were 0.899, -0.045, and 0.995, respectively.

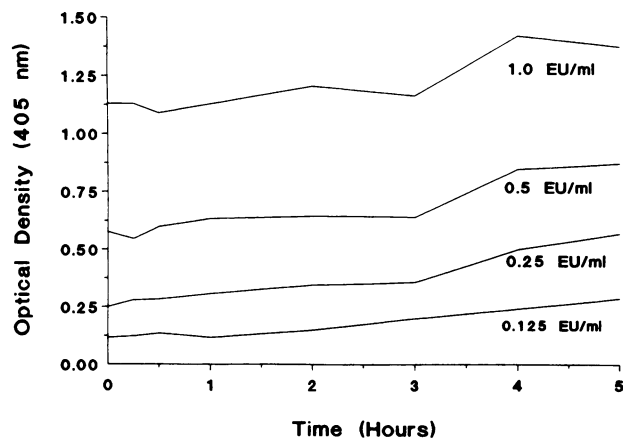


FIG. 2. Stability of the chromogenic LAL reagent. A set of standards, 0.125, 0.25, 0.5, and 1.0 EU/ml, was assayed over a 5-h time span with the chromogenic LAL reagent and the endpoint method. The same vial of lysate was used for all of the tests and was maintained on ice and in the light.

coefficients of variation of these curves were all below 7% (Table 1).

Stability. A series of standards assayed during a 5-h period remained constant for the first 3 h. The LAL was kept on ice but in the daylight. The same endotoxin dilutions were used in each test. Over the first 3 h, the slopes of these curves varied from 1.090 to 1.189 and the y intercepts varied from -0.03 from -0.08. The standard lines for the various time intervals were parallel but not identical because of the increase in the background color of the LAL with time.

These data can be plotted as the optical density of a single endotoxin concentration with time (Fig. 2). While the optical density increased slightly with time, the change was not great and there was no pronounced rise or fall in the first 3 h of the test.

Sensitivity. The sensitivity or the detection limit of the method depends on both the time required to activate the enzyme and the time allowed for color development of the substrate. With the single-reagent test, the lowest concentration of endotoxin used was 0.125 EU/ml, and this was detected in 45 min.

Endpoint method with multiple reagents. With a multiple-reagent chromogenic method in which the enzyme is activated by LAL in the first step and color is developed after addition of the buffer and the substrate in the second step, the sensitivity can be increased to 0.0005 EU/ml by increasing the incubation time. A test with endotoxin standards ranging from 1.0 to 0.125 EU/ml required 15 min for the first step and 3 min for the second. When the time allowed for step 1 was increased to 45 min and 3 min was maintained for the second step, the sensitivity was increased to 0.01 EU/ml. With initial incubation for 60 min and 5 min for color development, the sensitivity was increased to 0.05 pg/ml or 0.0005 EU/ml (Fig. 3).

Kinetic method with chromogenic LAL reagent. In the kinetic chromogenic assay, the increase in A_{405} was plotted against time for a range of endotoxin concentrations (Fig. 4). These curves were distinct from one another, and while the base line or negative control had a constant rise, it was easily separable from the 0.03-EU/ml endotoxin concentration.

The standard line (Fig. 5) was achieved by plotting the log of the endotoxin concentration against the log of the T_o (i.e., time to reach 100 milliabsorbance units). This standard line

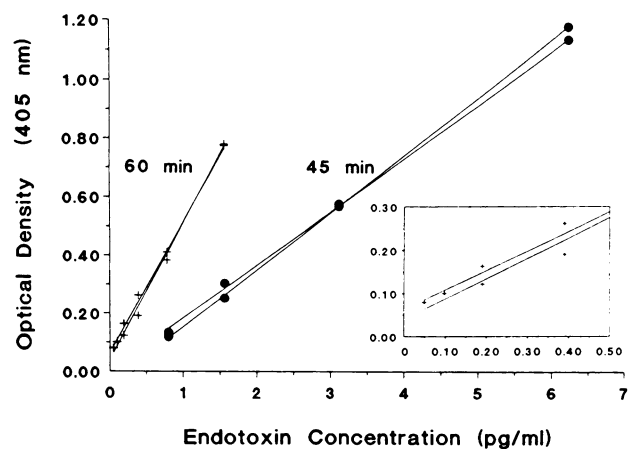


FIG. 3. Sensitivity of a multistep endpoint chromogenic method. With a multistep endpoint chromogenic test, a range of endotoxin concentrations from 6.25 to 0.05 pg/ml was assayed. In the 45-min test, endotoxin and LAL were incubated for 45 min, followed by incubation for 3 min with the substrate. The 60 min of incubation with endotoxin and LAL was followed by 5 min of incubation with the substrate for maximum color development. The insert represents an enlargement of the data from 0 to 0.5 pg of endotoxin per ml.

was run in duplicate and had an r value of 0.9978. The results of unknowns run in the same microplate with the standards are shown in Table 2. The coefficients of variation for the two unknowns were 13 and 9.9%.

In three experiments run on three different days, the equations for the standard line were very similar. The slopes ranged from -0.171 to -0.179, the y intercepts ranged from 1.807 to 1.831, and the r values were all 0.99 or greater.

This test was also simultaneously run in glass tubes and in polystyrene flat-bottom microplates. There was very little difference between the final optical densities obtained in glass tubes and those obtained in microplates (data not shown).

Kinetic method with gel clot-chromogenic LAL reagent. A single-reagent kinetic chromogenic LAL can also be prepared by mixing gel clot LAL with Spectrozyme and a

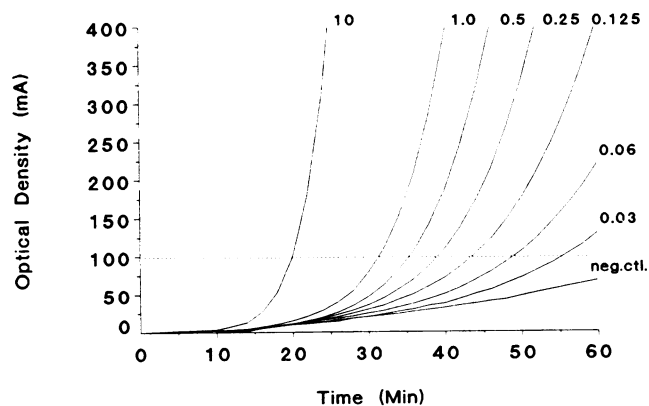


FIG. 4. Chromogenic kinetic test. Increase in optical density with time for a range of endotoxin concentrations (10 to 0.03 EU/ml) with the chromogenic LAL reagent. The dotted line at 100 milliabsorbance units (mA) represents the T_o abscissa. neg.ctl., Negative control.

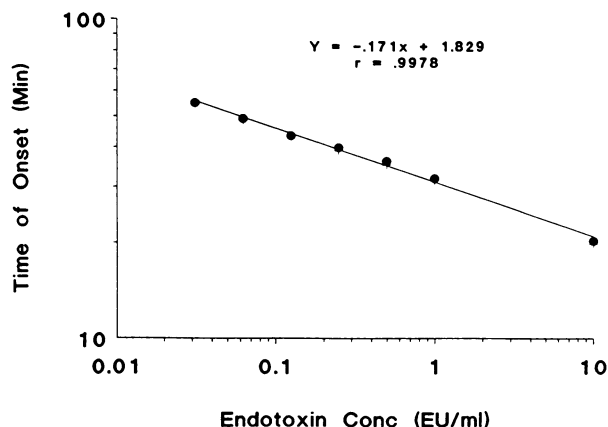


FIG. 5. Standard line for the chromogenic kinetic method using 100 milliabsorbance units as the T_o . The log of the T_o is plotted against the log of the endotoxin concentration (Conc).

buffer. For Fig. 6, the increase in optical density with time was plotted for each endotoxin concentration. While the base line has an upward drift, the curves generated were distinct from each other and the background. The standard line had a slope of -0.163 , a y intercept of 1.802 , and a correlation coefficient of 0.9939 . Replicates of unknowns measured with this standard are shown in Table 2.

DISCUSSION

A number of drawbacks to the LAL assay remain, despite numerous modifications designed to improve quantitation, sensitivity, speed, and precision. Foremost among these is the limited range of sensitivity (about 1 order of magnitude) with either the turbidimetric or the chromogenic fixed incubation time (endpoint) method. A second drawback to endpoint methods is the requirement that an operator must end or read the reaction at a precise time. In the chromogenic test, acid must be added to stop the test. The optical density can be read in a spectrophotometer at any time thereafter. In the endpoint turbidimetric method, the reaction cannot be stopped without destroying the turbidity but must be read immediately after a specific incubation period.

The kinetic turbidimetric LAL assay provided several improvements over endpoint methodology. Since the kinetic turbidimetric method used a single reagent and did not require operator attention after initiation of the test, preci-

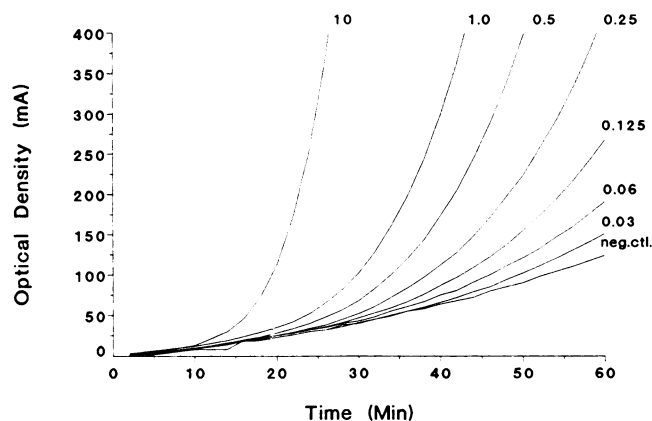


FIG. 6. Chromogenic kinetic test. Increase in optical density with time for a range of endotoxin concentrations with the gel clot-chromogenic LAL reagent. mA, Milliabsorbance units; neg.ctl., negative control.

sion, speed, and accuracy were all improved. Most importantly, the range of the test was increased from 1 to greater than 5 orders of magnitude. However, turbidity determinations made with a spectrophotometer use the decrease in transmitted light caused by physical blocking, and thus Beer's law does not apply. Particle size and number and reflected and refracted light all affect measurement to various degrees. Elegant electronic filtering and computer smoothing of data have been successfully used (9, 12), but these methods are available only on specialized optical readers. In addition, some products must be diluted to be assayed with the kinetic turbidimetric method.

In an attempt to resolve some of the problems associated with both the endpoint and kinetic turbidimetric methods, a kinetic chromogenic method was devised. Because of different reaction optima for the endotoxin-LAL activation step and the LAL-chromogenic substrate reaction, a previous attempt using a kinetic chromogenic method met with limited success (17). These researchers suggested that the method was not successful because there was competition between the chromogenic substrate and coagulogen for the activated enzyme. In the system described here, a single LAL formulation was found which permitted both an endpoint test and a kinetic test.

To check whether turbidity development interfered with the chromogenic readings, absorbance was remeasured after addition of 0.05 ml of 50% acetic acid immediately after the final reading was taken during a chromogenic kinetic test. Although slightly lower readings were obtained, the slight turbidity represents only minor interference with the test results (data not shown).

In our comparison of the endpoint and kinetic chromogenic methods, the linearity of standard lines was equally good. When the equations of these standard lines were used to calculate replicate unknown samples, the resulting coefficients of variations were all lower than 10%. Similar samples tested with a kinetic turbidimetric assay were as good or better (data not shown).

The sensitivity of the endpoint method using the chromogenic LAL reagent is adequate for water samples, and the method can detect 0.125 EU/ml with a 45-min incubation period. This long incubation period can be attributed to the pH of the lysate and some inhibition of the cascade by the substrate (data not shown). The pH of the chromogenic LAL reagent is 8.5, which is not optimal for either the enzyme

TABLE 2. Intravial reproducibility of kinetic chromogenic method

Endotoxin concn (EU/ml) ^a	Mean \pm SD endotoxin concn (coefficient of variation [%]) measured with:	
	Chromogenic LAL reagent	Gel clot-chromogenic LAL reagent
1.0 (4)	1.0159 \pm 1.014 (9.98)	
0.125 (6)	0.126 \pm 0.0167 (13.17)	
0.0 (6)	ND ^b	
0.25 (9)		0.22 \pm 0.0146 (6.64)
0.06 (6)		0.07 \pm 0.0018 (2.46)
0.0 (15)		ND

^a Numbers in parentheses are numbers of samples.

^b ND, Not detected.

cascade or substrate cleavage but permits both reactions. The kinetic method using the single reagent is more sensitive than the endpoint method using the chromogenic LAL reagent and can detect 0.03 EU/ml in 60 min.

The sensitivity of a chromogenic endpoint method can be increased by using multiple reagents and providing an optimal pH for both the cascade and the substrate reaction. The endpoint test with multiple reagents detected 0.125 EU/ml in 15 min and as little as 0.0005 EU/ml if incubated for 60 min and allowed to react with the substrate for 5 min.

One major drawback to colyophilized reagents is a tendency to form insoluble complexes or at least powders that are difficult to solubilize. For our combined lyophilized reagent, brief sonication was required to effect complete solution. Without sonication, variable results were obtained. This solubility problem can be avoided by using the gel clot-chromogenic reagent prepared by mixing gel clot LAL, a buffer, and a chromogenic substrate in the correct proportions. This reagent, which can be used for both endpoint and kinetic methods, is clear and well dispersed. This type of single-step chromogenic reagent has been used with variable success in other endpoint methods with different chromogenic substrates (1, 4, 11).

In conclusion, while both kinetic and endpoint tests were successfully performed with the chromogenic LAL reagent, all of the expected advantages of a kinetic chromogenic method over the kinetic turbidimetric method were not realized. The base line in the kinetic chromogenic method rises during the 60-min test, limiting resolution to 0.03 EU/ml. The chromogenic LAL reagent is a complicated formulation which requires sonication for even dispersion and is stable for only 3 h after reconstitution.

On the other hand, kinetic methods, either turbidimetric or chromogenic, have definite advantages over endpoint methods. These include extended range and sensitivity, precision, ease of operation, and less operator involvement. Further work is in progress to develop a more advantageous single-step chromogenic LAL reagent.

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