Plasminogen Activator Activity and Plasma-Coagulum Lysis Measured by Use of Optimized Fibrin Gel Structure Preformed in Microtiter Plates

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We introduce a new fibrin plate assay performed in microtiter plates. By means of spectroscopic studies we optimized the structure of the fibrin gel and then used the optimized fibrin gel to determine plasminogen activator activity. Plasminogen activator solutions were applied on top of the fibrin gel, and the absorbance of the gel was recorded at 405 nm. After incubation for 17 h at 25 °C, the absorbance was measured again. The difference in absorbance was proportional to the concentration of plasminogen activator, such that the dose–response curves were linear when the difference in absorbance was plotted as a function of the logarithmic concentration of plasminogen activator. We assayed both tissue-type and urokinase-type plasminogen activator activity. The intraassay CV was <4.7% (n = 20); the interassay CV was <3.1% (n = 15). Using the optimized procedure, we modified the assay for determination of plasma-coagulum lysis time in human plasma. We established a reference interval for lysis time in apparently healthy subjects of 75 to 201 ks. Patients with deep vein thrombosis showed significantly (P = 0.013) higher values.

Indexing Terms: fibrinolysis/urokinase/enzymatic methods/clotting/thrombosis/reference interval

The conversion of plasminogen to plasmin is catalyzed by plasminogen activators. The activity of plasminogen activators can be estimated in vitro by determining the amount of plasmin generated by their action. The recommended procedure for determining the biological activity of plasminogen activators is a clot lysis assay (1, 2). Two different procedures are commonly used. The conventional clot lysis assay is characterized by a uniform distribution of plasminogen activators in the fibrin substrate, whereas the fibrin plate assay makes use of a preformed fibrin gel, on top of which the plasminogen activator solution is placed (3).

The conventional clot lysis assay is difficult to automate and has inherent problems, of which poor precision and poor sensitivity are the most prominent. The introduction of turbidimetric clot lysis assays performed in microtiter plates (4–6) has improved the performance of the assay. However, the lysis profiles obtained by these methods demand many readings of absorbance and the use of computer programs to determine the exact time of lysis. Moreover, the sensitivity of these assays is lower than that of the standardized fibrin plate assay (7), but the fibrin plate assay is time consuming and laborious and is used only in the specialized laboratory.

Using the principles of the fibrin plate assay, we have developed a turbidimetric, sensitive, and precise microtiter plate assay with high capacity. The application of plasminogen activator on top of a fibrin gel preformed in microtiter plates facilitates a linear breakdown of the gel. Therefore, only a few readings of absorbance are necessary to determine the fibrinolytic activity of the applied sample.

Previous reports have shown that the physical and biochemical structure of a fibrin gel depends on the polymerization conditions—among which the concentrations of fibrinogen, thrombin, and calcium (8–10) and the conductivity of the fibrinogen solution are of particular importance (11). The presence of macromolecules such as dextran, polyethylene glycol (12, 13), and poloxamer 188 (14) modifies the structure of fibrin, and these reports have also shown that the lytic efficacy of the plasminogen activators depend on the structure of the fibrin. By means of in-house and commercially available fibrinogen, we studied the conditions for fibrin gel formation and incorporated these findings in the standardization of our assay. We found evidence that the fibrin structure is important for the analytical performance.

According to recent international recommendations (International Federation of Clinical Chemistry, International Union of Pure and Applied Chemistry, International Society on Thrombosis and Haemostasis), the present terminology of “euglobulin clot-lysis time” should be changed to “plasma-coagulum lysis; time (coag, diss, procedure)” (P-Coagulum lysis), and the results should be expressed in kiloseconds (ka) (15). With results of our optimized procedure we determined P-Coagulum lysis and we defined detection limit, imprecision, analytical performance, and reference values. We demonstrated that the assay can be used to separate patients with spontaneous venous thrombosis from healthy control subjects.

Materials and Methods

Reagents

Barbital buffer, pH 7.75, contained, per liter, 50 mmol of sodium barbital, 32 mmol of CaCl₂, and 1.6 mL of Tween 80 (polyoxyethylene sorbitan monooleate).

Tris buffer, pH 7.75, contained, per liter, 50 mmol of Tris, 21 mmol of CaCl₂, and 1.06 mL of Tween 80.

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Sample buffer I, pH 7.75, contained, per liter, 50 mmol of sodium barbital and 1.0 mL of Tween 80. For clinical samples this buffer was supplemented with 2.7 mmol/L K₂EDTA.

Sample buffer II, pH 7.75, contained, per liter, 50 mmol of Tris and 1.06 mL of Tween 80.

Lyophilized bovine thrombin (EC 3.4.21.5), 5000 NIH units (Leo Pharmaceuticals, Copenhagen, Denmark), was reconstituted with 50 mL of 0.15 mol/L NaCl to give a stock solution containing 0.1 NIH unit/L. This solution was stored at −20 °C until use. Thrombin working solution, containing 0.001 NIH unit/L, was prepared by diluting the stock solution with 0.15 mol/L NaCl containing 1.0 mL/L Tween 80. Bovine plasminogen was obtained from American Diagnostica, Greenwich, CT. Dextran (T10, T40, T70, and T500) were obtained from Pharmacia Biotech, Sollentuna, Sweden. Polystyrene microtiter plates were from Nunc, Roskilde, Denmark.

Bovine fibrinogen was prepared in-house according to Brakman (16); this stock solution was divided into 10-mL aliquots and kept at −70 °C in polystyrene tubes. Before use the fibrinogen solution was thawed at 37 °C and centrifuged at 2000 g for 20 min at 20 °C. The supernate was filtered through glass wool to remove unsedimented content. The fibrinogen concentration was 41.3 μmol/L, determined as described elsewhere (17). The fibrinogen preparation contained 13.6 μmol/L plasminogen, determined as reported by Mussoni et al. (18), and 87% of the protein content was clottable. The conductivity was 25.0 mS cm⁻¹, determined with a conductivity meter (Radiometer, Copenhagen, Denmark). We prepared a fibrinogen working solution with a pH of 7.75 and a conductivity of 6 mS cm⁻¹ by diluting the stock solution with the barbital buffer and H₂O. The working solution contained, per liter, 3 μmol of fibrinogen, 1 μmol of plasminogen, 20 mmol of Ca²⁺, and 1.0 mL of Tween 80.

Commercially available bovine fibrinogen (prod. no. 462) was obtained from American Diagnostica. One vial of fibrinogen was reconstituted in 2 mL of H₂O, giving a fibrinogen concentration of 52.9 μmol/L; 97% of the protein was clottable, and the conductivity was 20.5 mS cm⁻¹. We prepared a fibrinogen working solution with a pH of 7.75 and a conductivity of 6 mS cm⁻¹ by diluting the stock solution with Tris buffer enriched in plasminogen. The working solution contained, per liter, 3 μmol of fibrinogen, 1 μmol of plasminogen, 20 mmol of Ca²⁺, and 1.0 mL of Tween 80.

Plasminogen Activators

Human tissue-type plasminogen activator (t-PA; EC 3.4.21.68) from Bowes melanoma cell culture, standardized against WHO reference preparation lot no. 83/517, was purchased from Biopool, Umeå, Sweden. Low-molecular-mass urokinase-type plasminogen activator (u-PA; EC 3.4.21.73) from human urine, standardized against WHO reference preparation lot no. 87/594, was obtained from American Diagnostica. Both plasminogen activators were reconstituted according to the manufacturer, further diluted in sample buffer I or II, and kept on crushed ice until use.

Reference Material and Clinical Samples

Fifty healthy subjects (26 women and 24 men, median age 30.1 years) served as the reference population. The subjects were evaluated as healthy after an extensive interview. Twenty-five consecutive patients (13 women and 12 men, median age 33.6 years) with spontaneous deep vein thrombosis were included in the study. Informed consent was obtained from all the subjects, and the procedures followed were in accordance with the ethical standards of our responsible committee.

Blood samples were collected between 0800 and 0900 into siliconized evacuated tubes (Becton Dickinson, Meylan, France) according to recommended procedures (19). The samples were rapidly frozen at −70 °C and kept stored at this temperature. Before analysis the samples were thawed at 37 °C for 5 min and placed on crushed ice.

A pool of plasma from another 30 healthy subjects, collected and prepared as just described, was also analyzed in each series of samples.

Plasma euglobulin fractions were prepared as previously reported (7).

Assay Procedures

For experimental studies. We added 20 μL of thrombin working solution and 200 μL of fibrinogen working solution to the wells of a microtiter plate, covered the plate with adhesive tape, and placed it horizontally at 25 °C for at least 4 h to obtain a stable fibrin gel. We then carefully placed 30 μL of the sample (plasminogen activator) on the fibrin surface and measured the absorbance (A₀) of the fibrin and activator solution at 405 nm with a Titertek Twinreader (Flow Laboratories, Irvine, UK). The measurement is controlled by means of the ELISA+ software from Meddata (Doylestown, PA). After sealing the plate with adhesive tape and incubating for 61.2 ks (17 h) at 25 °C, we measured the absorbance again (Aₐ₁₂). The decrease in absorbance (ΔA) was proportional to the concentration of plasminogen activator in the applied sample.

For reference material and clinical samples. Duplicate 30-μL portions of the plasma euglobulin fraction are carefully placed on top of the preformed fibrin gel prepared as described above, and the A₀ values of the fibrin and sample solutions are recorded. The plate is covered with adhesive tape and incubated at 25 °C. The absorbance is again read after 61.2, 72.0, and 86.4 ks (Aₐ₁₂, Aₐ₂₀, and Aₐ₈₆.₄ respectively), corresponding to 17, 20, and 24 h of incubation.

Results

Fibrin Gel Studies

Optimization of the fibrin gel structure. Spectroscopic evaluation of the fibrin structure was performed by measuring the absorbance at 405 nm of fibrin gels,
prepared from in-house and commercially available fibrinogen, kept for 6 h at 25 °C after thrombin had been added to the fibrinogen solution.

We used a fibrinogen concentration of 3 μmol/L and varied the concentration of thrombin between 0.01 and 100 × 10⁻⁵ NIH units/L. The relation between thrombin concentration and absorbance is shown in Fig. 1A. We also determined the relation between fibrinogen concentration and absorbance in fibrin gels prepared with increasing concentration of fibrinogen (Fig. 1B).

Increasing amounts of CaCl₂ (0–25 mmol/L) added to the fibrinogen solution resulted in an absorbance plateau when the calcium concentration exceeded 15 mmol/L (Fig. 1C).

We also varied (by adding NaCl) the conductivity of the fibrinogen solution from 6 to 23 mS⁻¹; the results are shown in Fig. 1D.

To summarize the findings in Fig. 1, the optimal conditions for formation of coarse fibrin require, per liter, 0.001 NIH unit of thrombin, 3 μmol of fibrinogen, and 20 mmol of Ca²⁺, and a conductivity of 6 mS⁻¹.

As we varied the pH of the fibrinogen solution from 7.45 to 8.5, the conductivity of the fibrinogen solution was virtually unaltered, and only minor fluctuations in absorbance were observed (results not shown).

**Effect of added dextran.** We added 0–30 g/L of four types of dextran (T10, T40, T70, and T500) to in-house and commercially available fibrinogen solutions, using 200 μL of these fibrinogen preparations and 20 μL of thrombin at two different concentrations, 0.1 and 0.001 NIH unit/L, to prepare fibrin gels with low and high absorbances. When the dextran was incorporated in the gels prepared with 0.1 NIH unit/L thrombin, there was a pronounced increase in absorbance. In contrast, the absorbance of the fibrin gel with a thrombin concentration of 0.001 NIH unit/L was unchanged (data not shown).

**Effect of plasminogen concentration.** We added 0–5 μmol/L of plasminogen to in-house and commercially available fibrinogen and prepared fibrin gels as previously described and used these plasminogen-enriched fibrin gels to analyze two concentrations (1250 and 310 U/L) of t-PA and u-PA. The fibrinolytic activity of both plasminogen activators increased as a function of the concentration of plasminogen (data not shown). We therefore enriched the commercially available fibrinogen with plasminogen to a final concentration of 1 μmol/L, i.e., the same concentration as in the working solution of in-house fibrinogen.

**Effect of fibrin structure on calibration curves.** Fibrin with high absorbance was prepared as described in Materials and Methods. Fibrin with low absorbance was prepared by using 200 μL of fibrinogen (3 μmol/L) and 20 μL of thrombin (0.1 NIH unit/L). We diluted t-PA and u-PA in sample buffer to yield concentrations ranging from 10 to 10 000 U/L. Sample buffer I was...
used for samples analyzed on fibrin prepared from in-house fibrinogen, sample buffer II for samples analyzed on fibrin prepared from commercially available fibrinogen. We placed 30 μL of the plasminogen activator solutions on top of the fibrin gel and measured the activity of the plasminogen activators as described in Assay Procedures. We observed a linear relationship between the logarithm of the concentration and the fibrinolytic activity of the plasminogen activator in the concentration ranges of 80–2500 U/L for t-PA and 200–2500 U/L for u-PA (Fig. 2). The correlation coefficients exceeded 0.998 when fibrin with high absorbance was used (n = 20) and exceeded 0.996 for fibrin with low absorbance (n = 20). However, the use of fibrin with low absorbance decreased the slope of the calibration curves (Fig. 2).

**Stability of the fibrin gel.** The velocity of the conversion of fibrinogen to fibrin is dependent on the concentration of thrombin. For a thrombin concentration of 0.001 NIH unit/L, incubation of fibrinogen and thrombin for 4 h was necessary to obtain a uniform and stable fibrin gel. Prolongation of the incubation period beyond 4 h gave no further increase in absorbance.

To investigate the potential for spontaneous lysis of the fibrin, we incubated the fibrin gel at 25 °C for 7 days in the microtiter plate sealed with adhesive tape. We saw no sign of spontaneous lysis, an observation we validated by repeatedly measuring absorbance at 405 nm. However, samples determined on fibrin gels kept for this long showed increased activity. Therefore, fibrin gels to be used for analyses in the afternoon were prepared in the morning.

**Intermethod Comparison of Lysis Profiles**

Different patterns for the breakdown of the fibrin gel were obtained with a conventional clot lysis assay as compared with the present assay. For the conventional clot lysis assay, we added 30 μL of t-PA solution (1250 U/L) to 20 μL of thrombin solution (0.001 NIH unit/L), followed without delay by 200 μL of in-house fibrinogen preparation (3 μmol/L). The lysis profile was obtained by repeated measurements of the absorbance of the fibrin gel at 405 nm. The initial part of the conventional clot lysis profile was characterized by a steep increase in absorbance: This illustrated the action of thrombin on fibrinogen, i.e., the formation of the fibrin gel. The increase was followed by an absorbance plateau, which was followed by breakdown of the fibrin gel and an abrupt decrease in absorbance (Fig. 3).

The microtiter fibrin plate assay showed a quite different pattern. After 30 μL of t-PA (1250 U/L) was placed on top of the preformed fibrin gel, there was initially no detectable change in absorbance. This lag period was followed by a continuous decrease in absorbance, illustrating a slow but linear breakdown of the fibrin gel (Fig. 3).

The clot lysis profiles for u-PA showed similar differences between the two assays (data not shown).

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**Fig. 2.** Effect of concentrations of t-PA (A, B) and u-PA (C, D) on assays with fibrin gels prepared from in-house (solid lines) and commercially available fibrinogen (dotted lines).

(A, C) coarse fibrin with high absorbance; (B, D) fine fibrin with low absorbance.

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Analytical Performance

Imprecision. Adding Tween 80, 1.0 mL/L, when preparing the fibrin gels produced a pronounced decrease of imprecision in \( A_0 \); 1.087 ± 0.009 (mean ± SD; CV = 0.8%, n = 60) vs 1.007 ± 0.081 (CV = 8.0%, n = 60). Moreover, without Tween 80, the plasminogen activator solution was not dispersed over the fibrin surface, but remained as hemispheric drops on top of the fibrin layer, providing erroneous results. Addition of Tween 80 solely to the sample buffer did not reduce the imprecision (CV = 7.6%, n = 60).

We investigated the intraassay and interassay imprecision of the method and compared the results obtained with fibrin of two different structures prepared from in-house fibrinogen as previously described. Initially we studied the imprecision of the slope of the calibration curves obtained with both types of plasminogen activators. For the fibrin with low absorbance the intraassay CV of the slope of the calibration curve obtained with t-PA was 4.5% (n = 12); this decreased to 1.1% when fibrin with high absorbance was used. The corresponding values for u-PA were 3.2% and 1.5%, respectively (n = 12). The interassay CV for t-PA was 5.3% on fibrin with low absorbance, decreasing to 2.1% when fibrin with high absorbance was used (n = 15). The corresponding values for u-PA were 3.9% and 1.8%, respectively (n = 15).

We also evaluated the CV of two concentrations of plasminogen activators, 1250 and 310 U/L. The imprecision was always lower when the activity was determined on fibrin with high absorbance, compared with fibrin with low absorbance (Table 1).

The imprecision of P-Coagulum lysis was examined on the pooled plasma from 30 healthy subjects. The intraassay CV was 4.2% (n = 30), the interassay 6.9% (n = 10).

Detection limit. We determined the detection limit of the assay as the concentration of plasminogen activator that reduced \( A_0 \) by 3 SD of the \( A_0 \) mean; this corresponds to a \( \Delta A \) of 0.027. The detection limits for t-PA and u-PA were 66 U/L and 190 U/L, respectively.

Reference Material and Clinical Samples

The lysis profile obtained with plasma euglobulin fractions showed a pattern similar to that obtained with t-PA (Fig. 3), i.e., a lag phase followed by a linear breakdown of the fibrin gel. We used two corresponding values of time (ks) and absorbance to construct the line determining the linear part of the clot lysis profile for the individual samples, and we extrapolated this line to the time of total lysis of the fibrin gel (corresponding to \( A = 0.18 \)) and read the P-Coagulum lysis in ks according to the international recommendation (15).

For clinical samples we determined the absorbance at time 0, 61.2, 72.0, and 86.4 ks to secure that both the readings used for the calculation were positioned on the linear part of the curve; i.e., the absorbances did not represent either the lag phase or complete lysis of the fibrin gel. In 73 of the 75 samples these criteria were fulfilled for the measurements \( A_{72.0} \) and \( A_{86.4} \). One sample from a patient had a \( \Delta A \) below the detection limit of the assay (0.027; corresponding to 595 ks). For statistical evaluation we expressed this sample as 595 ks. In one sample from a subject in the reference group, the \( A_{86.4} \) reading indicated complete lysis, so we used \( A_{81.2} \) and \( A_{72.0} \) to calculate P-Coagulum lysis. The results are shown in Fig. 4.

In the reference population we observed a gaussian distribution of P-Coagulum lysis: range 74–230 ks, mean 138 ks, median 133 ks, and SD 31.7 ks. The resulting reference interval was 75–201 ks (mean ± 2 SD). However, the distribution of the data from the patients with deep vein thrombosis was not gaussian, but ranged from 88 to 308 ks (+ one outside the detection limit) (median 163.1). Use of the Mann–Whitney rank sum test to compare the difference in P-Coagulum lysis between the reference subjects and the diseased subjects showed a significantly longer P-Coagulum lysis time in the latter group (\( P = 0.013 \)). The area under a receiver operating characteristic curve has been reported to be a good measure of the accuracy of a clinical test (20). Therefore we constructed such a curve on the basis of the results of P-Coagulum lysis and calculated the area under the curve to be 0.7. This indicates that our test has a moderate accuracy.

Table 1. Analytical imprecision of assay of t-PA and u-PA.

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<th>Interassay (n = 15)</th>
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<td>2.0</td>
<td>11.4</td>
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<tr>
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<td>1.4</td>
<td>6.8</td>
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<td>1.3</td>
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Fig. 3. Lysis profiles obtained with 30-μL t-PA (1250 U/L) samples in the conventional clot lysis assay (Y) and the microliter fibrin plate assay (X).

The absorbance of the fibrin gels was repeatedly measured from 0 to 79.2 ks. For details see text.

<table>
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be effective in the production of coarse fibrin (12, 13), this observation suggests that, under the present experimental conditions, we have reached the upper limit of the absorbance of the fibrin used in our assay. Thus, our preformed fibrin gel has a structure characterized by a high susceptibility to the action of plasminogen activators.

The concentration of plasminogen is reported to alter the fibrinolytic activity of plasminogen activators when measured with the fibrin plate assay (7) and with turbidimetric clot lysis assays (4, 5, 22–24). In concordance with these observations, we found that the activity of plasminogen activators depends on the concentration of plasminogen, and a fixed amount of plasminogen is important to secure reproducible results. Our results stress that the design of biological assays for the standardization of plasminogen activator activity should thus include a standardization of the plasminogen concentration.

We compared the clot lysis profile obtained with a conventional clot lysis assay with that obtained with the microtiter fibrin plate assay. We confirmed that the conventional clot lysis assay demands continuous registration of the lytic process to determine the exact time of lysis (4–6). In contrast, only few measurements of absorbance were necessary to construct the clot lysis profile with the microtiter fibrin plate assay. The slope of the linear part of the curve depended on the concentration of plasminogen activator to provide a linear relationship of the dose–response curves for t-PA and u-PA, and we found that the dose–response curves for the two plasminogen activators showed excellent linearity when ΔA was plotted as a function of the logarithm of the concentration of plasminogen activator, i.e., as an exponential function. The slope of the calibration curves was much higher when we used fibrin with high absorbance instead of fibrin with low absorbance. This finding supports that the response of the plasminogen activators is greatest on fibrin with a coarse structure.

In our studies of imprecision we found that incorporating Tween 80 in the fibrin gel was important to obtain a low CV. The imprecision of the calibration curves (slope) and two different concentrations of t-PA and u-PA imprecision was always lower for the fibrin with high absorbance (coarse fibrin) than for the fibrin with low absorbance (fine-stranded fibrin). For plasma samples the intra- and interassay CVs of the P-Coagulum lysis determinations were 4.2% and 6.9%, respectively.

With the assay conditions described, the detection limit of the assay was 66 U/L for t-PA or 190 U/L for u-PA. However, prolonging the incubation period to 40 h or increasing the temperature to 37 °C improved the detection limits to 10 U/L for both types (data not shown).

Previously reported assays for determining P-Coagulum lysis have failed to produce a reference interval based on a gaussian distribution (27). In contrast, with the present assay we observed a gaussian distribution.

Discussion

The fibrin plate assay was introduced four decades ago by Astrup and Müllertz (3) and is still in use (7). The principle of the present assay is that plasminogen activator solutions are applied on the top of a preformed fibrin gel in microtiter plates. The ΔA at 405 nm after incubation at 25 °C is proportional to the concentration of the plasminogen activator in the applied sample.

In developing the microtiter fibrin plate assay we have paid particular attention to optimization of the fibrinolytic substrate, i.e., fibrin, because variation in the fibrin gel structure substantially affects the lytic efficacy of plasminogen activators (21–26). We obtained identical results for in-house and commercially available fibrinogen diluted in barbital buffer and Tris buffer, respectively. In addition, the structure of the fibrin gel depends on the conditions under which fibrin is formed, being strongly influenced by the concentrations of thrombin, fibrinogen, and calcium, as well as the conductivity of the fibrinogen solution, whereas pH is of minor importance. Optimizing the reaction conditions with respect to these factors yielded a fibrin gel with a high absorbance; such a gel is characterized by a coarse structure with a high mass/length ratio (9). We were unable to make the fibrin gel coarser (i.e., exhibit higher absorbance) by adding dextrans to the fibrinogen solution. Because dextrans have been reported to

![Figure 4: P-Coagulum lysis time (ks) for 50 healthy subjects (reference population) and 25 patients with deep vein thrombosis. Mean and 2 SEM are indicated for each population.](image-url)
in a healthy population and identified a reference interval from 75 to 201 ks (Fig. 4). However, the distribution of the data from the patients with vein thrombosis was not gaussian, and the data were significantly higher than for the reference population \((P = 0.013)\).

At present, no convenient international calibrator exists for assessing the P-Coagulum lysis. Therefore, careful standardization of the analytical conditions, as reported in our study, is of utmost importance. Our method can be applied in nonspecialized laboratories without sophisticated equipment, is suitable for the standardization of the activity of plasminogen activator preparations, and can be used in the clinical laboratory for determination of P-Coagulum lysis.

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