Simple, Sensitive Method for Measuring Plasmin and Plasminogen Activity in Plasma

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We describe a sensitive, simple, and rapid method for measuring plasmin and plasminogen in plasma, with fibrinogen as substrate. The assay can be done within 1 h. Plasma is diluted 20-fold with buffer and 0.1 ml (5 μl of original plasma) is incubated for 5 min at 37 °C, with or without 400 units of streptokinase. Then 2.0 ml of fibrinogen solution is added, the mixture again incubated (37 °C, 5 min), and the reaction stopped. The amount of tyrosine liberated from the fibrinogen is measured and is related to activity. This method is suitable for routine clinical work if the same batch of fibrinogen is used.

Additional Keyphrases: substrate specificity • normal (reference) values • activation with streptokinase • fibrinogen as substrate • intermethod comparison

Plasmin (EC 3.4.21.7) is an enzyme known to have broad specificity (1, 2). Accordingly, many rate-determination methods for measuring the proteolytic activity of plasminogen and plasmin in blood have been reported, with use of various substrates. Fibrinolytic or caseinolytic methods are now being widely used. The popular fibrin plate method developed by Astrup and Mullertz (3) provides much useful information, but this method is semiquantitative and time-consuming. Radio- or fluorescent-labeled fibrin and fibrinogen (4–9) are also used for the determination, requiring special labeled preparations. Methods in which fibrin or fibrinogen are used are poorly reproducible, because most fibrinogen and thrombin preparations cannot be prepared without variation from batch to batch. The caseinolytic method (10–15) is simple and reproducible from laboratory to laboratory. The Subcommittee on Standardization of the National Heart Institute’s Committee on Thrombolytic Agents has developed a method in which α-casein is used, which is now being recommended for general use (15), but a question still remains as to whether or not α-casein is the most suitable substrate. Results by esterolytic methods of measuring the activity of plasmin in plasma, with use of synthetic substrates such as p-tosyl-L-arginine methyl ester, do not parallel the results obtained by procedures based on proteolytic activity, indicating that the synthetic substrates are less specific than fibrinogen or casein (16).

In addition to such rate-determination measurement of proteolytic activity, direct methods for measuring plasmin and plasminogen in blood have been recently developed, such as radioimmunoassay and affinity chromatography (17–19). The first is the most sensitive and specific method, but 72 h is required for the determination; the second requires chromatographic technique. Various immunochemical methods have also been reported (20–24).

Here, we report a sensitive, simple and rapid method for measuring plasmin and plasminogen by using fibrinogen, the physiological substrate for plasmin.

Materials and Method

Materials

Fibrinogen preparation. We used the following bovine fibrinogen preparations: MIN 5285, clottable protein 60% (Armour Pharmaceutical Co.); 51C-2870, Fraction I, Type I, clottable protein 65% (Sigma Chem. Co.); Lot 60, clottable protein 60% (Miles Lab. Inc.); Product No. 44039 (BDH Chem. Ltd., England); Lot 96, clottable protein 63.6% (Poviet Production, Netherland).


Streptokinase, “Varidase” (no. EC number assigned) Lederle Div., American Cyanamide Corp.


Trypsin. Difco.


Tris(hydroxymethyl)aminomethane buffer, 0.1 mol/liter, pH 7.5; phosphate buffer, 0.1 mol/liter, pH 7.5; and trichloroacetic acid, 150 g/liter, Nakarai Chem. Co., Japan.


Procedure

Preparation of plasma specimens: Immediately after venous blood was drawn, nine parts of blood were mixed with one part of an aqueous solution of 36 g of sodium citrate per liter, cooled, and centrifuged (10000 × g, 15 min, 0°C). The plasma was either used immediately or stored at −20°C.

Enzyme assay: For the plasminogen assay, plasma specimen was diluted 20-fold with 0.1 mol/liter tris(hydroxymethyl)aminomethane buffer, pH 7.5; 0.1 ml of the diluted plasma specimen (5 μl of original plasma) was immediately mixed with 400 units of streptokinase in 0.1 ml of water and incubated at 37°C for 5 min. Two milliliters of fibrinogen solution (clottable protein, 36 g/liter of 0.1 mol/liter tris(hydroxymethyl)aminomethane buffer, pH 7.5) was then added, and the mixture was incubated at 37°C for 5 min. (All fibrinogen preparations of Armour, Miles, Sigma, BDH, and Poviet can be used.) After incubation, 1.0 ml of the trichloroacetic acid solution was added and mixed well to stop the reaction. Control tubes were prepared in the same way, except that the trichloroacetic acid solution was added before the fibrinogen. The tyrosine liberated from fibrinogen was measured by use of the Folin–Ciocalteau phenol reagent (10). After centrifugation (3000 rpm, 10 min) the supernate was filtered through Toyo filter paper No. 6, and 0.5 ml of the filtrate was mixed with 0.6 ml of phenol reagent diluted three-fold with water. Two milliliters of NaOH (0.5 mol/liter) was then added, and 15 min later the absorbance was measured at 660 nm. Tyrosine standard solution prepared in fibrinogen solution was similarly treated and used to calculate the tyrosine liberated from fibrinogen substrate by enzyme action.

Plasmin activity was assayed without treating the plasma specimen with streptokinase. Diluted plasma specimen was incubated directly with 2.0 ml of fibrinogen solution at 37°C for 5 min, and treated as described above.

Definition of enzyme activity: One unit of enzyme activity is defined as the activity by 1 ml of plasma that liberates 1 nmol of tyrosine from fibrinogen in 1 min at 37°C.

Results

Effect of pH on fibrinogenolysis, fibrinolysis, and caseinolysis in plasma treated with streptokinase. Proteolytic activity of plasma or serum treated with streptokinase was maximal at about pH 7.5, with use of fibrinogen, fibrin, or casein.

Effect of the concentration of fibrinogen, fibrin, and casein on streptokinase-activated proteolytic activity in plasma. When the concentration of fibrinogen was increased, the proteolytic activity in plasma was increased, leveling off at a concentration of over 30 g of clottable protein per liter with the fibrinogen preparations of Armour or Miles. The optimal concentration of fibrin and casein for the determination of proteolytic activity in plasma or serum was more than 6–8 g/liter under the conditions of the present assay.

Effect of various concentrations of streptokinase on the activation of plasminogen in plasma. At streptokinase concentrations exceeding 200 units/0.1 ml of plasma or serum, plasminogen was maximally activated.

Effect of time on the activation of plasminogen by streptokinase. Some reports (11, 16) have indicated that the time needed for complete activation of plasminogen is less than 2 min, but others (12, 14) have shown that 5 min is necessary. In this study, for maximal activation of plasminogen in plasma or serum by streptokinase, a 5-min incubation period was necessary.

Relationship between incubation time or quantity of serum and observed enzyme activity in plasma

Fig. 1. Relationship between incubation time or quantity of serum and observed enzyme activity in plasma

Fibrinogenolytic activity was determined as described here. For the fibrinolytic or caseinolytic activity, plasma specimen was mixed with streptokinase (400 units per 0.1 ml of plasma) and incubated at 37°C for 5 min. Fibrin or casein solution (clottable protein, 0.8 g/100 ml; 0.1 mol/liter tris(hydroxymethyl)aminomethane buffer, pH 7.5), 4 ml, was then added and incubated at 37°C for 10 to 30 min. After incubation, 1.0 ml of 200 g/liter trichloroacetic acid solution was added to stop the reaction. Control tubes were prepared in the same way, except that the trichloroacetic acid was added before fibrin or casein. The amount of tyrosine liberated from fibrin or casein was determined as described here.
Table 1. Proteolytic Activity of Streptokinase-Activated and Non-Activated Plasma Specimens, Trypsin, and Pronase

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Plasma + streptokinase</th>
<th>Trypsin, Pronase</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>400 units/0.1 ml</td>
<td>300 μg/50 μg</td>
</tr>
<tr>
<td>Fibrinogen (Miles)</td>
<td>6.6 2482</td>
<td>0.9</td>
</tr>
<tr>
<td>Casein (Hammersten)</td>
<td>2.4 6.3</td>
<td>30.6 13.3</td>
</tr>
</tbody>
</table>

Fibrinogenolytic activity studied under conditions described in the experimental procedure. Caseinolytic activity determined by using 0.1 ml of plasma, 300 μg of trypsin, and 50 μg of pronase, under conditions described in Figure 1. Each result is averaged data from three experiments.

ml, the fibrinolytic and caseinolytic activities were also linearly correlated with the amounts of plasma or the incubation time for as long as 20 min at 37 °C (Figure 1).

Effect of NaCl on the fibrinogenolytic activity in plasma. In the recommended method of the National Heart Institute's Committee on Thrombolytic Agents, with α-casein as substrate, a 60 mmol/liter tris(hydroxymethyl)aminomethane buffer containing 90 mmol of NaCl per liter is used (15). Therefore, we studied the effect of ionic strength on enzymatic activity. The fibrinogenolytic activity of plasma in 0.06 to 0.1 mol/liter tris(hydroxymethyl)aminomethane or phosphate buffer was greater than in the same concentration of these buffers containing 45 to 90 mmol of NaCl per liter. No significant difference was observed between the fibrinogenolytic activity obtained with use of tris(hydroxymethyl)aminomethane buffer and phosphate buffer.

Evaluation of fibrinogen and casein as substrates for measuring proteolytic activity in blood. Because various proteolytic enzymes are present in blood, the specificity of various substrates for measuring plasmin activity in blood was studied. The streptokinase-activated plasma digested fibrinogen more efficiently than casein, whereas the proteolytic activities of trypsin (EC 3.4.21.4) and pronase (EC 3.4.21.4) were more significant with casein than fibrinogen (Table 1). When the streptokinase-activated proteolytic activity in blood was studied with fibrinogen, fibrin, and casein as substrates, the fibrinogenolytic activity was the most marked, and it can be measured with use of 5 μl of plasma or serum and a 5-min incubation at 37 °C, while for the determination of fibrinolytic or caseinolytic activity 0.1–0.3 ml of plasma or serum and a 20-min incubation were necessary (Figure 1). Under the conditions of the present method, with 5 μl of plasma and a 5-min incubation, no caseinolytic activity of plasma was observed.

Comparison of various fibrinogen substrates for measuring plasmin and plasminogen activity. Hydrolysis of fibrinogen from Armour, BDH, or Poviet by plasmin in plasma or serum was greater than that of the fibrinogen preparation of Miles or Sigma (Figure 2). Liberation of tyrosine from each of these fibrinogen preparations and the amounts of plasma used or the incubation times were linearly related (Figure 2). Furthermore, results obtained with the fibrinogen from Armour, Miles, or Poviet correlated well with each other (Figure 3).

Effect of streptokinase on the fibrinogen preparation. We saw no proteolytic effect of streptokinase on the fibrinogen preparations of Armour, Miles, Sigma, BDH, or Poviet.

Effect of Transamin and Trasylol on the fibrinogenolytic activity of plasma. The specific plasmin inhibitors, Transamin (5 × 10⁻² mol/liter) and Trasylol (500 units per tube), inhibited the streptokinase-activated fibrinogenolytic activity in plasma under the conditions of the present assay method, suggesting that this fibrinogenolytic activity is due to plasmin and plasminogen.

Effect of acidification of plasma on fibrinogenolytic activity. The method of Alkjaersig et al. (25) was used. One part of plasma or serum was mixed with one part of 0.16 mol/liter HCl, left at room tempera-
ture for 15 min, then neutralized with one part of 0.16 mol/liter NaOH and diluted 20-fold with 0.1 mol/liter tris(hydroxymethyl)aminomethane buffer, pH 7.5. Fibrinogenolytic activity was then assayed, and found to be increased by an average of 10% in 26 of 50 plasma specimens; however, 22 of the 50 specimens showed an average 10% decrease in total fibrinogenolytic activity (Figure 4).

**Correlation between results obtained by present fibrinogen method and method with fibrin plate (Hyland's "Enzo-Diffusion" fibrin plate) or casein.** We studied plasma specimens from healthy subjects, pregnant women, and patients with various diseases such as liver cirrhosis and cancers. A good correlation was observed between the results obtained by the present method with Miles' fibrinogen preparation and the fibrin plate method with Hyland's Enzo-Diffusion fibrin plate, indicating reliability of the present method (Figure 5). The fibrinogenolytic activity studied by the present method was also correlated with the caseinolytic activity (n = 97, r = 0.5455, P < 0.01).

**Effect of meals on plasmin and plasminogen activity in plasma.** The effect of food intake on the fibrinogenolytic activity in plasma was an increase in the enzymatic activity in 1 to 2 h after a meal, an ef-

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**Table 2. Plasmin and Plasminogen Activity in Plasma of Normal Persons and of Pregnant Women or Patients with Various Diseases**

<table>
<thead>
<tr>
<th>No. cases</th>
<th>Plasmin Tyrosine liberated, nmol/ml/min (37 °C)</th>
<th>Plasmin/Plasminogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal subjects</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>10</td>
<td>224 ± 205</td>
</tr>
<tr>
<td>Female</td>
<td>10</td>
<td>93 ± 125</td>
</tr>
<tr>
<td>Thrombosis</td>
<td>14</td>
<td>176 ± 153</td>
</tr>
<tr>
<td>Liver diseases</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver cirrhosis</td>
<td>9</td>
<td>155 ± 174</td>
</tr>
<tr>
<td>Acute hepatitis</td>
<td>11</td>
<td>119 ± 174</td>
</tr>
<tr>
<td>Chronic hepatitis</td>
<td>5</td>
<td>100 ± 83</td>
</tr>
<tr>
<td>Banti's disease</td>
<td>3</td>
<td>20 ± 91</td>
</tr>
<tr>
<td>Cancer</td>
<td>15</td>
<td>70 ± 70</td>
</tr>
<tr>
<td>Pregnancy, 32–40 weeks</td>
<td>20</td>
<td>193 ± 212</td>
</tr>
</tbody>
</table>

Plasma specimens obtained between 0900 and 1000 hours were assayed by use of fibrinogen preparation of Miles. Results are expressed as means ± SD.
fect clearly observed in plasma, but not in serum. However, this effect of meal intake was not so clearly observed with regards to caseinolytic activity.

**Plasmin and plasminogen activity in plasma in various diseases.** The plasminogen activity in pregnant women was markedly increased, but in liver diseases, particularly in liver cirrhosis, the plasminogen activity was markedly decreased (Table 2). Plasma obtained from patients with thrombosis showed slightly increased concentrations of plasminogen. The ratio of plasmin to plasminogen was markedly increased in cases of liver cirrhosis, even though plasminogen activity was low. On the other hand, this ratio was within normal limits in pregnant women, although the plasminogen activity was markedly increased.

**Discussion**

The present method for measuring the plasmin and plasminogen activity by use of fibrinogen is more specific than is the caseinolytic method. In a comparison of the specificity of fibrinogen and casein for measuring the plasmin activity, the streptokinase-activated proteolytic activity in plasma was more markedly observed with fibrinogen than with casein, whereas the proteolytic activities of trypsin and pronase were more marked with casein than fibrinogen. Under the conditions of the present method (5 μl of plasma and 5-min incubation), hydrolysis of casein was not observed, indicating that trypsin and other proteolytic enzymes in blood that hydrolyze casein more rapidly than fibrinogen do not digest fibrinogen. These observations clearly support the high specificity of the present method.

The present method is far more sensitive than any yet devised in which casein or the fibrin plate are used. A multi-sample run can be completed in less than an hour. Reproducibility of the present method is satisfactory, if the same batch of fibrinogen preparation is used. Under the conditions of the present method, and with use of fibrinogen preparations from various suppliers, the rate determination of plasmin and plasminogen activity in plasma was satisfactory. In this study, we used Miles' fibrinogen preparation for routine clinical work, because it is cheaper and we think that it is less likely to be contaminated by plasmin and plasminogen, although greater sensitivity was obtained with the fibrinogen preparations of Armour, BDH, and Poviet.

Reportedly (13, 14), untreated plasma or serum should not be used for plasminogen assay because plasmin inhibitors are present. The euglobulin fraction is used, which contains no plasmin inhibitors. Acid treatment of plasma has also been recommended, to inactivate plasmin inhibitors for the determination of plasmin (14), and the plasminogen values so obtained are reported to be identical to those for the euglobulin fraction (14). In this study, acid-treatment of plasma resulted in inconsistent values. Although plasminogen is reported to be resistant to acidification (14), this apparently is incorrect. In fact, we saw instances of decreased activity of plasminogen after acid treatment of plasma or serum. In addition, acidification has been reported not to eliminate completely the inhibitors (12). Therefore, it is doubtful whether or not acid treatment is the best way to inactivate plasmin inhibitors.

Macfarlane et al. (26, 27) showed that the clot dissolved more rapidly if the plasma was diluted before clotting, and they explained this on the basis of a dissociation of the inhibitor/enzyme complex on dilution. Therefore, in this study plasma was diluted before plasmin and plasminogen activity were determined. We incubated 0.05 to 0.2 ml of 20-fold diluted plasma (2.5 to 10 μl of original plasma) with 2 ml of fibrinogen solution and observed a linear relationship between fibrinogenolytic activity and enzyme concentration. However, this enzyme assay was still influenced to some extent by plasmin inhibitors. In fact, the acid treatment of plasma or serum increased the fibrinogenolytic activity by an average of 10%, but this increase in the value did not modify the clinical diagnostic significance in various diseases. Therefore, in this study we used diluted, non-acidiﬁed plasma specimens.

Clinically, the effect of food intake on fibrinogenolytic activity and diurnal variation in enzyme activity must be taken into consideration. Although no effect of meals on the caseinolytic activity has been reported (28), in this study an effect of meal intake on fibrinogenolytic activity in plasma appeared to be significant. Therefore, in this investigation the plasmin and plasminogen activity in various diseases was studied with plasma obtained between 0900 and 1000 hours.

Results obtained by the present method correlated well with results obtained by the fibrin plate method and by the casein method involving Hammersten β-casein, indicating the reliability of this method. In addition, our results for persons with various diseases agree well with the results reported by previous workers using different methods.

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**References**


