Faster Determination of Clottable Fibrinogen in Human Plasma: An Improved Method and Kinetic Study

Yuji Inada, Hiroshi Okamoto, Shozo Kanai, and Yutaka Tamaura

Clottable fibrinogen in human plasma was determined by measuring spectrophotometrically the increase in turbidity with time due to the fibrinogen–fibrin conversion with thrombin. From the maximal absorbance, \( A_{\text{max}} \), at 450 nm obtained 2 min or less after thrombin is added to plasma, we estimated the fibrinogen concentration in plasma of normal subjects and patients. Analysis of the rate of the absorbance increase yielded the \( K_m \) value, \( 1.6 \times 10^{-5} \) mol/liter, which closely agrees with the \( K_m \) of 1.2 \( \times 10^{-5} \) mol/liter obtained by analysis of the fibrinopeptides released from fibrinogen.

Additional Keyphrases: emergency procedure • \( K_m \) value • intra-vascular coagulation syndrome

Ellis and Stranksy (1) reported the first turbidimetric technique for determination of plasma fibrinogen by measuring the absorbance increase due to fibrinogen–fibrin conversion with thrombin. This turbidimetric fibrinogen test is well suited for clinical use because of its accuracy and simplicity, but it requires 20 min per sample. Other turbidimetric methods for fibrinogen include salting out with ammonium sulfate (2–5), incubation of plasma at 56 °C in sodium chloride solution to obtain precipitates (6), measuring thrombin-induced clotting time (7), and immunodiffusion assay (8, 9). Determination of protein of the fibrin clot formed from fibrinogen with thrombin (10–12) is the most direct method for estimating fibrinogen, but the procedure is relatively complicated and time consuming.

In the clinical laboratory, a faster analysis for fibrinogen in plasma is urgently needed to diagnose and treat disseminated intra-vascular coagulation syndrome (13). The present paper deals with a rapid determination of clottable fibrinogen in human plasma by an improved method of Ellis and Stranksy’s turbidimetric technique (1), and also with the kinetic study of the increase in turbidity due to fibrinogen–fibrin conversion by thrombin. With this method, determination of fibrinogen in human plasma is relatively rapid.

Methods

Human fibrinogen and thrombin were obtained from The Green Cross Corporation. The fibrinogen preparation was 89% clottable with thrombin. The isolated fibrinogen was dissolved in tris(hydroxymethyl)aminomethane/acid citrate–dextrose buffer (five volumes of a mixture of, per liter, 115 mmol of NaCl, 16 mmol of KCl, 23 mmol of tris(hydroxymethyl)aminomethane (pH 7.0), and 5 mmol of glucose and one volume of acid citrate–dextrose solution) (14). The concentration of fibrinogen was spectrophotometrically determined, with use of the molar absorptivity \( \epsilon = 5.1 \times 10^{-5} \) liter mol\(^{-1}\) cm\(^{-1}\) at 278 nm (15), which was calculated from \( a = 15.0 \) and the molecular weight of 340 000. Activity of the thrombin preparation was 13 NIH units/mg of protein.

Clottable fibrinogen was turbidimetrically determined as follows. Into a spectrophotometric cell (0.5-cm path length) containing 1.0 ml of tris(hydroxymethyl)aminomethane-HCl buffer (pH 7.0; 10 mmol, plus 40 mmol of NaCl per liter) were sequentially added, with stirring, 0.5 ml of plasma or fibrinogen solution and 0.1 ml of thrombin (143 NIH units/ml) in isotonic saline. The reaction mixture was immediately covered with Parafilm, mixed well, and its absorbance at 450 nm was recorded with a Cary recording spectrophotometer, Model 14M, until the maximum value, \( A_{\text{max}} \), was obtained. The reference cell contained no thrombin.

In the experiment for measuring the rate of absorbance increase (\( \alpha \)), the reaction system was the same as above, except that a lower concentration of thrombin, 6.8 NIH units/ml, was used. Absorbance change at 450 nm was recorded with time.

Human blood was taken from normal subjects and patients, and anticoagulated with acid citrate–dextrose or 38 g/liter sodium citrate, respectively. The ratio of the volume of blood and anticoagulant was 6/1 for acid citrate–dextrose or 9/1 for sodium citrate. Human plasma was obtained by centrifugation of anticoagulated blood at 3000 × g for 10 min.

The concentration of clottable protein in plasma was also determined by the clot weight comparison method (10) and by a turbidimetric method in which ammonium sulfate is used as a salting-out agent (6).

Results and Discussion

Plasma of hospitalized patients was analyzed for fibrinogen. Figure 1 shows the absorbance change that results from the turbidity produced when fibrinogen in plasma is polymerized by the action of thrombin. The absorbance increased markedly with time, becoming constant 1 or 2 min after the thrombin was added to the mixture of plasma and the buffer containing NaCl. A similar absorbance increase with time was obtained for isolated fibrinogen at various concentrations.

Figure 2 shows the plot of the values of \( A_{\text{max}} \) vs. concentration of fibrinogen isolated from human plasma. At low fibrinogen concentration, the \( A_{\text{max}} \) value increased linearly with concentration, but at concentrations exceeding 7 mmol/liter the value deviated slightly from linearity. From this plot, one can determine the concentration of fibrinogen by using the following equation:

\[
F(\text{g/liter}) = 15 \times A_{\text{max}} \times \text{[dilution factor, 10/9 or 7/6]}
\]

where F stands for the amount of fibrinogen in solution and...
Fig. 1. Absorbance change at 450 nm after addition of thrombin to plasma of hospitalized patients

To a mixture of 0.5 ml plasma and 1.0 ml of buffer was added 0.1 ml of thrombin (143 NIH units/ml) at room temperature. Blood was anticoagulated with a 38 g/liter solution of sodium citrate (9/1 by vol). Constant absorbance was taken as A_max. The fibrinogen in normal plasma gives A_max values ranging from 0.084 to 0.26 (cf. Table 1).

Table 1. Concentration of Fibrinogen in Plasma of Hospitalized Patients as Measured from A_max and from Turbidimetric Method (Ammonium Sulfate Precipitation)

<table>
<thead>
<tr>
<th>Cases</th>
<th>Sex</th>
<th>Fibrinogen, g/liter (NH₄)₂SO₄</th>
<th>A_max</th>
</tr>
</thead>
<tbody>
<tr>
<td>H.I.(a)*</td>
<td>F</td>
<td>13.4</td>
<td>11.4</td>
</tr>
<tr>
<td>C.S.(b)</td>
<td>F</td>
<td>13.5</td>
<td>10.8</td>
</tr>
<tr>
<td>M.U.</td>
<td>M</td>
<td>9.8</td>
<td>8.2</td>
</tr>
<tr>
<td>A.K.(c)</td>
<td>F</td>
<td>10.0</td>
<td>8.0</td>
</tr>
<tr>
<td>T.M.(d)</td>
<td>M</td>
<td>10.5</td>
<td>7.0</td>
</tr>
<tr>
<td>I.M.(e)</td>
<td>M</td>
<td>8.6</td>
<td>5.8</td>
</tr>
<tr>
<td>S.T.(f)</td>
<td>F</td>
<td>10.2</td>
<td>4.7</td>
</tr>
<tr>
<td>F.O.(g)</td>
<td>M</td>
<td>8.0</td>
<td>4.3</td>
</tr>
<tr>
<td>K.U.(h)</td>
<td>F</td>
<td>4.6</td>
<td>3.6</td>
</tr>
<tr>
<td>T.I.</td>
<td>F</td>
<td>4.2</td>
<td>3.2</td>
</tr>
<tr>
<td>S.S.</td>
<td>F</td>
<td>3.1</td>
<td>3.2</td>
</tr>
<tr>
<td>S.K.(i)</td>
<td>M</td>
<td>4.7</td>
<td>2.9</td>
</tr>
<tr>
<td>Y.E.</td>
<td>F</td>
<td>4.1</td>
<td>2.4</td>
</tr>
<tr>
<td>M.Y.(j)</td>
<td>F</td>
<td>1.9</td>
<td>2.3</td>
</tr>
<tr>
<td>M.H.</td>
<td>F</td>
<td>3.9</td>
<td>2.2</td>
</tr>
<tr>
<td>N.S.(k)</td>
<td>F</td>
<td>1.7</td>
<td>1.8</td>
</tr>
<tr>
<td>K.F.(1)</td>
<td>F</td>
<td>1.7</td>
<td>1.2</td>
</tr>
</tbody>
</table>

* See corresponding symbols in Figure 1.

0.26  
0.1  
0.084  
0.04

Fig. 2. Maximum absorbance, A_max, as a function of concentration of fibrinogen isolated from human plasma

Closed circles: values obtained after adding 0.1 ml of thrombin (143 NIH units/ml) to a mixture of 1.0 ml of buffer and 0.5 ml of fibrinogen at various concentrations (0–10 g/liter). Ten grams of fibrinogen per liter of plasma corresponds to 9.19 µmol of fibrinogen per liter in the abscess of the figure. Open circles: values obtained from the same reaction system as above except that 0.1 ml of fibrinogen solution at various concentrations (0–30 g/liter) was added to 0.4 ml of plasma in a normal subject (clottable fibrinogen was 18 g/liter).

The dilution factor is the ratio of volume of blood and of anticoagulant.

To test whether fibrinogen in plasma can be accurately determined by using the above equation without any interference from plasma proteins, we did the next series of experiments. To a mixture of 1 ml of buffer and 0.4 ml of plasma containing 1.8 g of fibrinogen per liter as determined by the Fearnley-Chakrabarti method (10) was added 0.1 ml of known amounts of fibrinogen (0–30 g/liter). The total amount of fibrinogen in each sample solution was estimated by measuring A_max, which is shown by open circles in Figure 2. Observed values are on the linear portion of the standard curve, and analytical recovery of fibrinogen was almost complete (97 ± 10%). This suggests that no component in plasma interferes with the assay. Cannarozzi et al. (16) also reported that a high content of lipids, bilirubin, and fibrin split products in plasma does not affect Ellis and Stranasky’s turbidimetric assay (1).

Concentration of fibrinogen in plasma of hospitalized patients was determined from the values of A_max (Figure 1) by using the above equation, and also determined by the turbidimetric method based on precipitation with ammonium sulfate (5) as shown in Table 1. The fibrinogen concentration in plasma as obtained from A_max values was slightly lower than that from the precipitation method with ammonium sulfate, in which proteins other than fibrinogen may be precipitated. Because the mean fibrinogen concentration in normal subjects is about 3 g/liter, with a range of 1.4 to 4.4 g/liter (11, 16), A_max values for hospitalized patients ranging from 0.084 to 0.26 are normal.

The effect of thrombin concentration on the values of A_max was tested by adding thrombin to a fibrinogen solution or plasma. The A_max values were constant at more than 5 NIH units/ml in the reaction system. Therefore, the concentration of thrombin was fixed at 8.9 NIH units/ml in the present.
study. This decreases the time for the determination of fibrinogen and is probably sufficient to cause fibrinogen–fibrin conversion, even when anti-thrombin is present in the plasma. The determination of fibrinogen was most suitable when the NaCl concentration was 40 mmol/liter and the pH of the reaction system was 7.0. No effect of temperature between 10 and 30 °C was observed.

When a low concentration of thrombin (0.43 NIH units/ml) is used for the fibrinogen–fibrin conversion, the absorbance increase with time is slow and its rate, v, can be accurately estimated. The absorbance did not change for a while after thrombin was added, then increased linearly with time (Figure 3). The resulting curve was diphasic, which may reflect the relation between the enzymic activity and the substrate concentration. A double-reciprocal plot of values for v against fibrinogen concentration gave a straight line, which intersected the abscissa. The $K_m$ value of $1.6 \times 10^{-5}$ mol/liter obtained from this plot agrees closely with $K_m = 1.2 \times 10^{-5}$ mol/liter determined from the effect of fibrinogen concentration on the rate of release of fibrinopeptides from the fibrinogen molecule (17). From the coincidence of the $K_m$ values, the measurement of absorbance increase in the present study reflects fibrinogen–fibrin conversion by the action of thrombin.

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**References**