Improved Method for Measuring Fibrinogen in Plasma, with Use of a Plasmin Inhibitor

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We describe a modified procedure for quantitating plasma fibrinogen by use of the thrombin-clottable protein technique. The method is accurate and requires about 30 min to complete. During its formation and isolation, the clot is protected from the degradative action of plasmin by an inhibitor, ε-aminocaproic acid.

Because a quantitative fibrinogen determination is frequently requested in an emergency situation, the ideal method is one that not only is accurate, but also rapid.

The procedure of Ware et al. (1) as presented by Henry (2) meets the first requirement but is too time consuming for emergency use. Furthermore, if the patient’s fibrinolytic system has been activated, the clot formed in vitro would be degraded and a falsely low result obtained.

We have significantly modified the method of Henry (2). Like other authors (3, 4), we use ε-aminocaproic acid as a plasmin (EC 3.4.21.7) inhibitor. Time-saving steps have been introduced for dissolving the clot and quantitating the protein. The net result is a procedure that can be performed in about 30 min with no apparent loss of accuracy.

Materials and Methods

Instrumentation

All spectrophotometric readings were made with a Coleman, Jr. Spectrophotometer at 545 nm, with 19 × 150 mm cuvettes.

Reagents

The thrombin and biuret reagents have been described previously (2).

Clotting buffer. The directions given below should be followed exactly if precipitation of Ca₃(PO₄)₂ is to be avoided. With the aid of a magnetic stirrer, dissolve 0.91 g of KH₂PO₄ and 0.47 g of Na₂HPO₄ in about 850 ml of de-ionized water (phosphate concentration is 10 mmol/liter, pH 6.4). Dissolve 0.20 g of CaCl₂ in about 100 ml of de-ionized water. Reduce the speed of the stirrer to only that needed for adequate agitation and add the CaCl₂ solution. After about 30 s add 5.0 g of ε-aminocaproic acid (Sigma Chemical Co., St. Louis, Mo. 63178), 1.0 g of NaN₂, and 9.0 g of NaCl. Stir only long enough to dissolve the solid chemicals. If necessary, adjust the pH to 6.4 with dilute HCl or NaOH. Transfer, with rinsing, to a 1-liter volumetric flask and bring to volume. The reagent can be stored at room temperature and appears to be stable indefinitely.

Fibrinogen protein standard. This can be prepared by diluting the standard used for total protein determinations with saline (NaCl, 9.0 g/liter). A standard equivalent to a 3.0 g/liter fibrinogen concentration is considered ideal. Since 2.5 ml is used in the procedure, the stock protein standard is diluted to 1.20 g/liter. Stored at 4 °C, the solution is stable for at least six months.

Alkaline saline solution. Mix equal volumes of saline and a 60 g/liter NaOH solution.

Streptokinase solution, approximately 100 units/ml. A 10 000 unit “Varidase” oral tablet (Lederle Laboratories, Pearl River, N. Y. 10965) was ground up and mixed with 100 ml of saline. The mixture was allowed to stand at room temperature for about 2 h before use.


Pooled plasma. Blood that had been drawn into 5-ml “Vacutainer” tubes containing tripotassium ethylenediaminetetraacetate (Becton, Dickinson, and Co., Rutherford, N. J. 07070) was centrifuged at 2100 × g for 10 min. The plasma was aspirated, pooled, and either used within 1 h or divided into aliquots and frozen at −20 °C.

Procedure

Plasma anticoagulated with oxalate, citrate, or ethylenediaminetetraacetate may be used (2).

While the blood is being centrifuged, add 30 ml of clotting buffer to the required number of 50-ml Ehrenmeyer flasks. Pipet 1.0 ml of plasma (or 2.0 ml, if a low value is expected) into the appropriate flask and mix by swirling the flask. Add 0.20 ml of the thrombin reagent and mix thoroughly but gently. Allow the flask(s) to stand undisturbed for 15 min at room temperature.

During this time, warm about 3 ml of the protein standard to room temperature. Label 15-ml conical centrifuge tubes for the reagent blank, standard, control, and each patient. To the standard tube, add 2.5 ml of the protein standard and 2.5 ml of the 60 g/liter NaOH solution. Add 5.0 ml of the alkaline saline solution to the other tubes.

At the end of 15 min, the clots are isolated as follows. Tip the flask at about a 45 degree angle and lay a glass stirring rod onto the clot. Turn the rod slowly and move it down through the clot to wind the clot around it. Simultaneously, slowly decant the reaction solution into a 50-ml beaker. Decanting shortens the time required to wind the clot. Inspect the solution in the beaker to be certain that all the clot was isolated. Wash the glass rod and clot with a gentle stream of de-ionized water, and blot dry with a paper towel or filter paper. Insert the rod into the appropriate centrifuge tube.

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Table 1. Inhibition of Fibrinolytic Activity by \( \epsilon \)-Aminocaproic Acid

<table>
<thead>
<tr>
<th>Reagents added</th>
<th>Fibrinogen concn,(^a) g/liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. (a) 50 mg of ( \epsilon )-aminocaproic acid/liter</td>
<td>4.12</td>
</tr>
<tr>
<td>(b) 50 units of streptokinase(^b)</td>
<td></td>
</tr>
<tr>
<td>2. (a) No ( \epsilon )-aminocaproic acid</td>
<td>0</td>
</tr>
<tr>
<td>(b) 50 units of streptokinase</td>
<td></td>
</tr>
<tr>
<td>3. (a) 50 mg of ( \epsilon )-aminocaproic acid/liter</td>
<td>4.03</td>
</tr>
<tr>
<td>(b) No streptokinase</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Av of three determinations. 
\(^b\) Added to reaction solution before thrombin.

When all the clots have been isolated, turn off the heat under a boiling water bath (this is essential, because superheating at the bottom of the tube results in turbid solutions of badly denatured protein) and place all the centrifuge tubes into the water bath. Rotate the glass rods a couple of times during a 5-min warming, then remove the tubes from the water bath. Carefully tilt the sample tubes so that the solution runs toward the mouth, and rotate the glass rod briefly. This ensures that all fibrin is removed from the rod. Touch the bottom of the rod against the top inside wall of the tube for a few seconds to minimize fluid loss. Remove the rod from the tube. Add 1.0 ml of the biuret reagent to all the tubes and mix on a vortex-type mixer. Pour the solutions into appropriately labeled 19 \( \times \) 150 mm Coleman cuvettes. This step shortens cooling time. After 5 min, zero the instrument with the blank at 545 nm and record the absorbance (A) of the standard and test solutions. The fibrinogen concentration is calculated as follows:

\[
\text{Concn (g/liter)} = \frac{A \text{ (unknown)}}{A \text{ (standard)}} \times \text{standard value} \\
\text{ (g/liter)}
\]

If 2 ml of plasma were used, divide the final answer by 2.

Analytical Variables

Concentration of \( \epsilon \)-aminocaproic acid. After reviewing the work cited in references (3) and (4), we arbitrarily decided to include 5 mg of \( \epsilon \)-aminocaproic acid per milliliter of clotting buffer. To be certain that this concentration would inhibit fibrinolytic activity in human plasma, the procedure was performed in triplicate on pooled plasma under the conditions shown below. Streptokinase (no EC no. assigned) was added to activate plasminogen in the sample.

1. The buffer solution contained 5 mg of \( \epsilon \)-aminocaproic acid per milliliter, and 50 units of streptokinase was added before the thrombin reagent.
2. The buffer solution contained no \( \epsilon \)-aminocaproic acid, and 50 units of streptokinase was added before the thrombin solution.
3. The buffer solution contained 5 mg of \( \epsilon \)-aminocaproic acid per milliliter; no streptokinase was added.

The results are shown in Table 1.

Color development and linearity of the biuret reaction. The absorbance of the protein standard, treated as indicated above, was followed as a function of time after it was removed from the water bath and biuret reagent added. We found no significant increase in absorbance after the first 5 min.

Standard solutions ranging in concentration from 750 to 6000 mg/liter were prepared and run in triplicate through the procedure. Absorbance and concentration were linearly related.

Linearity of the procedure. To see if dilution and absorbance were linearly related, we diluted pooled plasma with saline (serum cannot be used, owing to the presence of thrombin and calcium) to produce \( \frac{1}{2} \), \( \frac{1}{4} \), and \( \frac{1}{8} \) dilutions. The procedure was performed in triplicate on these dilutions. The results are shown in Figure 1. The concentration range was from about 1.0 to 4.0 g/liter.

Modification for measuring extremely low fibrinogen concentrations. At extremely low fibrinogen concentrations, the clot formed from 1.0 ml of plasma is generally not isolated completely, because it lacks cohesiveness and tends to disintegrate when one tries to wind it around the glass rod. To establish experimental conditions under which extremely low fibrinogen concentrations could be quantitated, we assayed a plasma pool and then diluted it with saline to produce a fibrinogen concentration of about 1000 mg/liter. We then assayed this diluted plasma in triplicate, using 1.0 ml of plasma to establish the value. The plasma was then further diluted with saline to \( \frac{1}{2} \), \( \frac{1}{4} \), and \( \frac{1}{8} \) of this concentration. The minimum volume necessary to produce a well-formed clot was determined for these three dilutions. Using these minimum volumes, the diluted plasmas were assayed in triplicate. The values calculated based on dilution factor, the values actually obtained, and the minimum volumes necessary are shown in Table 2.

Within-run precision. The assay procedure was performed approximately 20 times on two plasma pools. One pool was diluted with saline to establish precision at the lower end of the normal range. The undiluted pool gave a mean value of 4.33 g/liter, a standard deviation of 64 mg/liter, and a coefficient of variation of 1.5%. The diluted pool gave a mean value of 1.74 g/liter, a standard deviation of 40 mg/liter, and a coefficient of variation of 2.9%.

Between-run precision. For routine quality control, an aliquot of a plasma pool is run with each analysis. The pool usually contains about 20 aliquots and lasts about three months. Results for one such pool, containing 17 samples and considered representative of the between-run precision of the method, were: mean value, 3.70 g/liter; standard deviation, 82 mg/liter; and coefficient of variation, 2.2%.

Accuracy of the method. We used a commercial fibrinogen standard (Hyland Laboratories, Costa Mesa, Calif. 92626) to assess the accuracy of the method. Sixteen vials...
from the same lot were analyzed during three months. The manufacturer's stated value was 3.30 g/liter. The mean value we obtained by our method was 3.25 g/liter.

Results and Discussion

The effect of plasmin on the fibrin clot unprotected by the presence of ε-aminocaproic acid was quite dramatic. At the end of the 15-min incubation period, only a few stringy pieces of gelatinous material could be seen in the flask. A 5 g/liter concentration of ε-aminocaproic acid is considered sufficient to inhibit plasmin and protect the clot.

The alkaline saline reagent was developed during the study of the linearity of the biuret reaction. Absorbance at zero dilution was closer to zero when the NaCl concentration was about the same in the protein standards and reagent blank, and the clot dissolves faster in this reagent than in 30 g/liter NaOH.

Henry (2) indicates that using ethylenediaminetetraacetate as an anticoagulant results in friable clots with poor tensile strength. In our experience, the quality of the clot obtained in this procedure was not influenced by the anticoagulant. It is also stated that adding the biuret reagent after dissolving the clot yields lower results. In this procedure the biuret reagent must be added after the tubes are removed from the water bath to avoid the appearance of a variable amount of CuO precipitate. The value obtained for the Hyland reference plasma argues against falsely low results caused by this change in technique. The fact that the standard and blank are subjected to the same conditions as the fibrin clot probably accounts for the elimination of this problem.

Trapping of plasma proteins in the clot has been discussed as a source of positive error (2). In theory, the greater the plasma dilution, the less trapping there should be. If a much larger volume of clotting buffer was used, the clot tended to fragment when we attempted to isolate it. If the volume was decreased to much less than 20 ml, the apparent fibrinogen concentration increased, as compared to values obtained when 30 ml of clotting buffer was used.

Foster et al. (5) reported turbidity in the final solution. They believed that this was produced by impurities in the thrombin reagent. We have not encountered this problem with patients' samples. Turbidity has developed during the assay of lyophilized material such as some lots of the Hyland product and the survey samples from the College of American Pathologists (also produced by Hyland). Extracting the solutions with diethyl ether significantly decreases the turbidity. We found that optimum results were obtained by extracting the standard and blank also.

Normal range. It seemed desirable to compare the normal range estimated by this method with the generally accepted range of 2.0 to 4.0 g/liter. Our normal-value studies were far from ideal because our hospital obtains blood extramurally from a regional blood bank and outpatient services are provided by another laboratory. Thus we used blood samples from patients being admitted for elective minor surgery, if review of the clinical history did not indicate any condition thought to influence fibrinogen concentrations. The samples were assayed only by our method. The mean value (n = 45) was 3.18 g/liter, and the standard deviation was 740 mg/liter. The most significant result is the fact that the mean value did lie near 3.0 g/liter.

The method presented was developed because a rapid method for quantitating fibrinogen was needed by clinicians in our institution, but we did not wish to sacrifice analytical capabilities for speed. Every attempt was made to keep the technique as simple as possible. The procedure has been in use over a year in our laboratory, and we find it to be simple, rapid, and reliable.

References


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**Table 2. Plasma Volume Increases to Quantitate Abnormally Low Fibrinogen Levels**

<table>
<thead>
<tr>
<th>Value expected&lt;sup&gt;a&lt;/sup&gt; (mg/liter)</th>
<th>Value found&lt;sup&gt;b&lt;/sup&gt; (g/liter)</th>
<th>Minimum plasma volume, ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>—</td>
<td>1100</td>
<td>1.0</td>
</tr>
<tr>
<td>820</td>
<td>780</td>
<td>2.0</td>
</tr>
<tr>
<td>550</td>
<td>550</td>
<td>2.0</td>
</tr>
<tr>
<td>270</td>
<td>280</td>
<td>4.0</td>
</tr>
</tbody>
</table>

<sup>a</sup> Calculated values based on dilution of plasma pool.

<sup>b</sup> Av of three determinations.