Comparison of Three Methods for Plasma Fibrinogen

ABDUS SALEEM, M.D., KAY FRETZ, and ARTHUR F. KRIEG, M.D.

Division of Clinical Pathology, The Milton S. Hershey Medical Center, The Pennsylvania State University, Hershey, PA 17033

ABSTRACT

Three plasma fibrinogen methods are presented based on (1) clottable protein assay by Biuret reagent, (2) measurement of clot absorbance and (3) thrombin time. These methods show good correlation and adequate precision on normal subjects as well as heparinized plasmas. The effects of interference owing to hemolysis, lipemia, bilirubin, heparin and fibrinogen degradation products are discussed briefly.

Introduction

Estimation of plasma fibrinogen is routinely requested for investigation and treatment of bleeding disorders. Methods commonly employed for estimation of plasma fibrinogen include (1) precipitation techniques based on salt fractionation, (2) thrombin time methods, (3) measurement of clot absorbance following addition of thrombin, (4) measurement of clottable protein following addition of thrombin and (5) immunological techniques with antifibrinogen serum. Salt fractionation techniques are rapid but are considered nonspecific and semiquantitative. Thrombin time methods are simple and rapid but are affected by heparin and fibrinogen degradation products. Clot absorbance methods may be insensitive in low fibrinogen range. Clottable protein methods appear relatively specific but are time consuming. Antifibrinogen sera cross-react with all the fibrinogen related antigens, and the procedure is too slow for emergency clinical situations. A modification of the Ellis and Stransky clot absorbance procedure and a modified thrombin time procedure were recently reported. This report compares these two procedures with Biuret method based on estimation of clottable protein.

Materials and Methods

REAGENTS

Polybrene-barbital buffer. Five mg of polybrene, 5.71 g of sodium barbital and 2.93 g of sodium chloride are dissolved in 900 ml deionized water, titrated with 1 N HCl (approximately 22.5 ml) to pH 7.2, and made up to 1,000 ml volume. This solution is stable for at least six months at room temperature.

Calcium chloride — 0.025M. Precisely 2.78 g of calcium chloride (anhydrous) are dissolved to 1,000 ml in deionized water. This solution is stable indefinitely at room temperature.

Thrombin—50 units per ml. One vial of
Topical Thrombin* containing 1,000 N.I.H. units of thrombin is reconstituted with saline to give 50 units of thrombin per ml. Aliquots are frozen in 0.5 ml quantities in 12 x 75 mm polystyrene tubes. This thrombin solution is stable indefinitely at —20°.

**Calcium-thrombin reagent.** Four volumes of 0.025M calcium chloride solution are added to one volume of 50 units per ml thrombin. This calcium-thrombin solution is prepared fresh before use, is kept on ice and is stable for one hour.

**Phosphate buffer (M/15, pH 6.4).** Solution A is made by dissolving 9.08 g potassium dihydrogen phosphate (KH₂PO₄) to 1000 ml in deionized water. Solution B is made by dissolving 11.88 g disodium phosphate (Na₂HPO₄·2H₂O) to 1000 ml in deionized water. The working solution is prepared by adding 73.2 ml of solution A to 26.8 ml of solution B. The pH is checked and adjusted if necessary to 6.4 ± 0.1.

**Saline.** Nine g of sodium chloride are dissolved to 1000 ml in deionized water.

**Heparin.**† Exactly 1000 units per ml. Appropriate dilutions are made in saline.

**Biuret reagent.** This is prepared as described by Henry et al.⁹

**Fibrinogen degradation products (FDP).** This is prepared according to the method of Mardar.¹² Quantitation of FDP is done by Thrombo-Wellcotest.®

**Lipemic Serum.** A lipemic serum with triglyceride concentration of 800 mg per dl was mixed with plasma pool in the following proportions: (1) lipemic serum: plasma pool = 1:3 (slight turbidity), (2) lipemic serum: plasma pool = 1:2 (moderate turbidity) and (3) lipemic serum: plasma pool = 1:1 (gross turbidity).

**Plasma pool.** A plasma pool was prepared from citrated plasma of 10 normal individuals and two ml aliquots frozen in 12 × 75 mm polystyrene tubes at —70°. Fibrinogen is measured on this plasma pool ten times by the Biuret method and the mean fibrinogen value is assigned to this pool.

**Normal subjects.** Fourteen healthy laboratory personnel (five male and nine female) donated blood for this study.

**Heparinized plasma.** Plasma obtained from another 13 healthy laboratory personnel (five male and eight female) was heparinized to obtain a final concentration of five units of heparin per ml of plasma.

**COLLECTION OF PLASMA**

Nine parts of blood are mixed with one part of 3.8 percent sodium citrate in a Vacutainer System® (B-D 3204 N). The blood is centrifuged at 1600 g for 10 minutes. Plasma is removed and stored at 4° until ready for use. The plasma so stored is stable for at least 24 hours.

**BIURET PROCEDURE**

This procedure is modified from those described by Ware et al.²⁰ and Foster et al.⁶

1. One ml of plasma is added to six ml of saline and three ml of phosphate buffer in a 25 ml flask.
2. Exactly 0.2 ml of thrombin (1,000 units per ml) is added to the diluted plasma. The clot is mixed and wound on an applicator stick.
3. The stick is removed from the flask and dipped in saline for five minutes with gentle agitation.
4. The stick with the clot is removed and is blotted dry with filter paper.
5. The clot is scraped off the stick into a mixture of 1.0 ml of Biuret reagent and 1.0 ml of 3 percent sodium hydroxide.
6. The 2.0 ml of reagent including clot is heated in a water bath at 56° until the clot is dissolved (30 to 60 minutes).
7. A blank is prepared with one ml of Biuret reagent plus one ml of 3 per-

---

* Parke-Davis
† Heparin sodium. Riker Laboratories Inc., Northridge, CA 91324
‡ Thrombo-Wellcotest. Burroughs Wellcome, Research Triangle Park, NC
TABLE I
Plasma Fibrinogen Measurements

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Normal Subjects</th>
<th>Heparinized Plasmas</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Range</td>
</tr>
<tr>
<td></td>
<td>n mg per dl</td>
<td>n mg per dl</td>
</tr>
<tr>
<td>Modified thrombin time</td>
<td>14 342</td>
<td>229 - 565</td>
</tr>
<tr>
<td>Modified Ellis Stransky</td>
<td>14 328</td>
<td>213 - 512</td>
</tr>
<tr>
<td>Biuret</td>
<td>14 361</td>
<td>263 - 584</td>
</tr>
</tbody>
</table>

Plasma Fibrinogen Measurements

In order to calibrate the unknown sample, steps 2 through 7 are followed and the results are read from the calibration curve.

MODIFIED THROMBIN TIME PROCEDURE

This procedure is described elsewhere in detail. Briefly, the procedure for the calibration curve is as follows:

1. The plasma pool is diluted 1:5, 1:10, 1:20 and 1:40 in polybrene buffer. The absorbance for each of these dilutions is measured according to steps 2 through 7.

2. Exactly 400 µl of polybrene barbital buffer are pipetted in each of two cuvets marked as blank and test.

3. Twenty µl of test sample are pipetted into blank and test cuvets. Each cuvet is covered with parafilm and mixed by gentle inversion.

4. Twenty µl calcium-thrombin reagent are added to the test, mixed by gentle inversion and a timer started.

5. Twenty µl of saline are added to the blank and mixed by gentle inversion.

6. At exactly 10 minutes, absorbance of the test is read against the blank at 340 nm.

7. The absorbance vs. the calculated fibrinogen value is plotted on linear paper and the line of best fit drawn.

MODIFIED ELLIS STRANSKY PROCEDURE

This procedure has been described elsewhere in detail. Briefly, the procedure for the calibration curve is as follows:

1. The plasma pool is diluted 1:1, 1:1.5, 1:2, 1:2.5 and 1:5 in Polybrene buffer. The absorbance for each of these dilutions is measured according to steps 2 through 7.

2. Exactly 400 µl of polybrene barbital buffer are pipetted in each of two cuvets marked as blank and test.

3. Twenty µl of test sample are pipetted into blank and test cuvets. Each cuvet is covered with parafilm and mixed by gentle inversion.

4. Twenty µl calcium-thrombin reagent are added to the test, mixed by gentle inversion and a timer started.

5. Twenty µl of saline are added to the blank and mixed by gentle inversion.

6. At exactly 10 minutes, absorbance of the test is read against the blank at 340 nm.

7. The absorbance vs. the calculated fibrinogen value is plotted on linear paper and the line of best fit drawn.

In order to calibrate the unknown sample, steps 2 through 7 are followed and the results are read from the calibration curve.

MODIFIED THROMBIN TIME PROCEDURE

This procedure is described elsewhere in detail. Briefly, the procedure for the calibration curve is as follows:

1. The plasma pool is diluted 1:5, 1:10, 1:20 and 1:40 in polybrene buffer.

2. Duplicate measurements are run on each dilution using the Fibrometer® system in which 0.2 ml of plasma pool dilution is incubated at 37° for 2 minutes; 0.2 ml of thrombin solution (50 units per ml) is then added and the clotting time determined.

3. The two clotting times obtained for each dilution are averaged.

4. The averages are plotted on double log paper against calculated fibrinogen values in mg per dl and the line of best fit drawn.

The unknown sample is diluted 1:20 with polybrene buffer. The procedure outlined in steps 2 and 3 is followed. The average is read from the calibration curve and multiplied by the dilution factor (× 20) to get a result in mg per dl.

It should be noted that once thawed, the thrombin solution should be kept on ice and used within the hour. If thrombin times are outside the calibration curve, a higher (1:40 or 1:80) or a lower (1:2 or 1:5) dilution of plasma in polybrene buffer may be used. Results from calibration curve are multiplied by the appropriate dilution factor.

Technicon Inc., Tarrytown, NY 10591
INTERFERING SUBSTANCES

The plasma pool is mixed with varying concentrations of hemoglobin, icteric serum, heparin, fibrinogen degradation products and lipemic serum. Plasma fibrinogen is performed by the three previously described methods before and after mixing with the interfering substance.

RESULTS

COMPARISON OF METHODS

Fibrinogen was measured on 14 normal subjects and 13 heparinized plasmas by the modified thrombin time, modified Ellis Stransky procedure and the Biuret method.
PLASMA FIBRINOGEN METHODS

Results are given in table I. Comparison of means by the t test\(^2\) shows that mean fibrinogen values by the three methods on normal subjects and heparinized plasmas are not statistically different at 1 percent level of confidence. In figures 1 and 2 are shown the comparison between modified thrombin time and modified Ellis Stransky procedures for normal subjects and heparinized plasmas. In figures 3 and 4 are shown the comparison between modified Ellis Stransky and Biuret procedures for normal subjects and heparinized plasmas. In figures 5 and 6 are shown the comparison between modified thrombin time and Biuret procedures for normal subjects and heparinized plasmas.

**COMPARISON OF PRECISION**

Day to day precision for the three methods is shown in table II, based on results for the plasma pool and the plasma pool diluted with saline. In table III is shown a comparison of precision by F test.\(^8\) For the undiluted plasma pool, the modified Ellis Stransky and modified thrombin time have significantly better precision than the Biuret procedure. For the diluted plasma pool, the

**TABLE II**

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Method</th>
<th>n</th>
<th>Mean mg per dl</th>
<th>SD mg per dl</th>
<th>CV * Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma pool (undiluted)</td>
<td>Modified thrombin time</td>
<td>20</td>
<td>320</td>
<td>11.5</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td>Modified Ellis Stransky</td>
<td>20</td>
<td>335</td>
<td>15.8</td>
<td>4.7</td>
</tr>
<tr>
<td></td>
<td>Biuret</td>
<td>20</td>
<td>340</td>
<td>34.1</td>
<td>10.0</td>
</tr>
<tr>
<td>Plasma pool (diluted)</td>
<td>Modified thrombin time</td>
<td>20</td>
<td>53</td>
<td>1.9</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td>Modified Ellis Stransky</td>
<td>20</td>
<td>46</td>
<td>5.5</td>
<td>12.0</td>
</tr>
<tr>
<td></td>
<td>Biuret</td>
<td>20</td>
<td>47</td>
<td>4.8</td>
<td>10.3</td>
</tr>
</tbody>
</table>

*Coefficient of variation.
TABLE III
Comparison of Day to Day Precision by the Three Methods

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Undiluted Plasma Pool F p</th>
<th>Diluted Plasma Pool F p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Modified thrombin time</td>
<td>1.8 &gt;0.05</td>
<td>11.7 &lt;0.01</td>
</tr>
<tr>
<td>vs. modified Ellis Stransky</td>
<td>4.5 &lt;0.01</td>
<td>1.4 &gt;0.05</td>
</tr>
<tr>
<td>Modified Ellis Stransky</td>
<td>8.2 &lt;0.01</td>
<td>8.6 &lt;0.01</td>
</tr>
<tr>
<td>vs. biuret</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Modified thrombin time has significantly better precision than either the modified Ellis Stransky or Biuret procedure.

Precision of duplicate measurements from normal subjects and heparinized plasmas is shown in table IV while in table V is shown a comparison of precision by F test. For both normal subjects and heparinized plasmas, the modified Ellis Stransky and modified thrombin time have significantly better precision than the Biuret procedure.

EFFECT OF INTERFERING SUBSTANCES

Hemoglobin. Moderately hemolysed samples (hemoglobin up to 150 mg per dl) do not interfere with any of the three fibrinogen methods. The modified Ellis Stransky method cannot be used with grossly hemolysed samples (hemoglobin of 350 mg per dl) since it is difficult to set the blank to 100 percent transmission. However, the modified thrombin time and Biuret procedure are not affected by gross hemolysis.

TABLE IV
Precision of Duplicate Measurement by the Three Fibrinogen Methods

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Normal Subjects (14) 1 CV</th>
<th>Heparinized Plasma (13) 1 CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Modified thrombin time</td>
<td>4.7</td>
<td>5.0</td>
</tr>
<tr>
<td>Modified Ellis Stransky</td>
<td>4.2</td>
<td>4.1</td>
</tr>
<tr>
<td>Biuret</td>
<td>10.0</td>
<td>10.7</td>
</tr>
</tbody>
</table>

Bilirubin. Bilirubin up to 22.0 mg per dl does not interfere with any of the three fibrinogen methods.

Heparin. No interference is noted in any of three methods at plasma heparin levels of 10 USP units per ml. The Ellis Stransky and Biuret procedures show decreased recovery of fibrinogen with heparin levels over 10 USP units per ml. The modified thrombin time method shows decreased recovery with heparin levels over 40 USP units per ml.

Fibrinogen degradation products. The Ellis Stransky method is not affected by levels up to 240 μg per ml. The modified thrombin time procedure is affected by FDP levels of 40 μg per ml, with decreased recovery of fibrinogen. The Biuret method is affected by levels of FDP of 240 μg per ml or more.

Lipemia. Lipemia does not seem to interfere with the modified thrombin time procedure. The Biuret procedure shows increased recovery of clottable protein with moderate to gross lipemia. The modified Ellis Stransky procedure cannot be run with gross lipemia, since it is difficult to set the blank to 100 percent transmission.

Discussion

The need for a rapid, simple, accurate plasma fibrinogen method cannot be over emphasized. A recent survey by the Center for Disease Control revealed wide interlaboratory variation for fibrinogen on a normal pool. The problem is compounded if in-
Interfering substances such as heparin, FDP, and lipemia are present.

In our hands, the three methods used show good correlation both in normal subjects and heparinized plasmas. The modified Ellis and Stransky method is a rapid microprocedure which does not appear affected by moderate hemolysis, bilirubin, heparin in therapeutic levels, fibrinogen degradation products or moderate lipemia. Samples showing gross lipemia or gross hemolysis cannot be analysed by this procedure as it may be difficult to set the blank to 100 percent transmission. The modified thrombin time procedure is rapid and not affected by hemolysis, lipemia, bilirubin or heparin in therapeutic levels. The procedure has a high precision in low range of plasma fibrinogen. However, fibrinogen degradation products (40 µg per ml or more) gave false low results. The Biuret procedure requires a relatively large sample volume (one ml). It does not seem to be affected by hemolysis, bilirubin or heparin in therapeutic doses. However, high levels of fibrinogen degradation products (240 µg per ml or more) give false low results while moderate to gross lipemia gives false high values. Precision of the Biuret procedure appears inferior to the modified Ellis Stransky or modified thrombin time method.

An ideal method for plasma fibrinogen has not yet been found. In our experience, however, the three methods presented are not too difficult for routine laboratory use and are useful clinically, provided that their limitations are clearly understood.

References