A Practical Method for the Determination of Fibrinogen

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THE SERIAL DETERMINATION OF FIBRINOGEN is a useful tool in the diagnosis, prognosis and management of various conditions such as acute myocardial infarction, rheumatic fever, gangrenous lesions and hepatic disorders (1). In obstetrical cases, particularly where hypofibrinogenemia may be the cause of gross bleeding in parturition, there is a need for a method which is accurate, simple to perform and reasonably rapid. The following method has been found useful and has the virtue of employing reagents in daily service in the laboratory.

PRINCIPLE

The fibrinogen in well-oxalated plasma is converted by the enzyme thrombin into fibrin. The clot is removed and hydrolyzed in the presence of alkali in a boiling water-bath. The amount of protein may be determined by the usual biuret reagent (2, 3), or, if the fibrinogen in the clot is below normal, by the more sensitive phenol-biuret reagent of Lowry et al. (4, 5).

REAGENTS

1. Dried double oxalate. 1.2 Gm. ammonium oxalate (NH₄)₂C₂O₄·H₂O and 0.8 Gm. potassium oxalate K₂C₂O₄·H₂O in 100 ml. water. Place 0.5 ml. of double oxalate solution into test tubes for each 5 ml. blood and rotate so that it is spread as a film on the walls. Dry in a stream of air, because it will decompose on heating.

2. Sodium chloride 0.9% solution. 9 Gm. per liter of water.
3. Thrombin topical. 100 NIH units/ml. It is convenient to dilute the entire contents of the vial up to 50 ml. with 0.9% saline and then divide it into 0.2 ml. aliquots in 15 × 125 mm. test tubes. Keep frozen until used.

4. Sodium hydroxide 1.0% solution. 10 Gm. NaOH per liter of water.

   a. Alkaline carbonate. Dissolve 20 Gm. Na₂CO₃ and 0.5 Gm. sodium or potassium tartrate (not Rochelle salt) in a liter of 0.1N NaOH.
   b. Copper sulfate. 1 Gm. CuSO₄·5H₂O per liter of water.
   d. Stock phenol reagent. Dissolve 100 Gm. of sodium tungstate (Na₂WO₄·2H₂O) and 25 Gm. of sodium molybdate (NaMoO₄·2H₂O) in 700 ml. of water in a 1500 ml. round-bottomed flask. Add 50 ml. of 85% phosphoric acid and 100 ml. of concentrated hydrochloric acid (sp. gr. 1.19). Mix thoroughly. Fit a reflux condenser to the flask (all-glass joints). Boil the solution gently for 10 hours. Remove the condenser, add 150 Gm. of lithium sulfate (Li₂SO₄) and wash down with 50 ml. of water. After solution is complete, carefully add 4-5 drops of 30% hydrogen peroxide and boil for 15 minutes to remove excess peroxide. If the solution is not clear and golden yellow in appearance, repeat the peroxide treatment. Cool the solution to room temperature, dilute to one liter, and filter if necessary through glass-wool into a glass-stoppered brown bottle. This reagent is stable for several months. Titrate this reagent with 0.1N NaOH to a phenolphthalein end-point. On the basis of this titration dilute the reagent with approximately the same volume of water to make it 0.9N in acidity. All glassware must be scrupulously clean as the method is extremely sensitive.

Biuret reagent. For full details and discussions of modifications of this reagent refer to Reinhold (2) and Friedman (3).

Standard protein. Solutions of the following, when checked by Kjeldahl for the nitrogen content may be used: Fibrinogen: (1) Bovine—commercially available of about 85% protein content. (The Warner-Chilcott Laboratory preparation was the best of those tested.) (2) Human—may be precipitated from pooled normal plasma as described under Procedure and used as a Standard.

Albumin. Bovine or human.
Table 1. Protein Standardization with Lowry-Phenol Reagent

<table>
<thead>
<tr>
<th>Dilute protein Soln. 1.0 mg./ml. mg.</th>
<th>Protein mg.</th>
<th>Water ml.</th>
<th>Equivalent Fibrinogen mg./100 ml. when 1 ml. plasma is analyzed*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05</td>
<td>0.05</td>
<td>0.15</td>
<td>125</td>
</tr>
<tr>
<td>0.10</td>
<td>0.10</td>
<td>0.10</td>
<td>250</td>
</tr>
<tr>
<td>0.15</td>
<td>0.15</td>
<td>0.05</td>
<td>375</td>
</tr>
<tr>
<td>0.20</td>
<td>0.20</td>
<td>0.0</td>
<td>500</td>
</tr>
</tbody>
</table>

* 100 ml. = 2500 × actual amount protein present, e.g., 0.05 mg.  
0.04 ml. aliquot protein × 2500 = 125 mg./100 ml. fibrinogen.

Serum. Normal, pooled.  
For Lowry-phenol method, prepare standard protein solution of 50 mg./100 ml. or 0.5 mg./ml. See Table 1.  
For biuret method, prepare standard protein solution of 0.25% or 250 mg./100 ml. See Table 2.

PROCEDURE

Draw 5 ml. of blood and put into tube containing 0.5 ml. of the dried double oxalate. Mix well to prevent the formation of clots. (Always keep the plasma-oxalate in the same proportion.)  
Centrifuge for 5 minutes, meanwhile thawing the number of thrombin tubes necessary for duplicate tests. Add 5 ml. of 0.9% sodium chloride rapidly to each tube and mix well. Add 0.5 or 1 ml. of plasma to each thrombin tube and mix quickly and thoroughly. Clotting seems to be complete in 5 minutes; although standing for one hour is recommended, no increase in clotting was found after 30 minutes in a number of plasmas tested.

Loosen the clot from the walls by shaking the tilted tube. Rim with

Table 2. Protein Standardization with Biuret Reagent

<table>
<thead>
<tr>
<th>Dilute protein Soln. 2.5 mg./ml. mg.</th>
<th>Protein mg.</th>
<th>Water ml.</th>
<th>Equivalent Fibrinogen mg./100 ml. when 1 ml. plasma is analyzed*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2</td>
<td>0.5</td>
<td>1.8</td>
<td>125</td>
</tr>
<tr>
<td>0.4</td>
<td>1.0</td>
<td>1.6</td>
<td>250</td>
</tr>
<tr>
<td>0.6</td>
<td>1.5</td>
<td>1.4</td>
<td>375</td>
</tr>
<tr>
<td>0.8</td>
<td>2.0</td>
<td>1.2</td>
<td>500</td>
</tr>
<tr>
<td>1.0</td>
<td>2.5</td>
<td>1.0</td>
<td>625</td>
</tr>
</tbody>
</table>

* 100 ml. = 250 × actual amount protein present e.g., 0.5 mg. protein × 250 = 125 mg./100 ml. fibrinogen.
a fine pointed glass rod if necessary, taking care that none of the clot adheres to the wall of the tube or the rod. Usually it can be removed completely in one large clot.

From the size of the clot judge which protein method is applicable. If below normal size, the Lowry method is preferable. If a poor clot is formed, centrifuge, and then wash the clot by centrifuging and decanting; add alkali for hydrolysis to the same tube to prevent loss. If hypofibrinogenemia is suspected, the test may be run on 2 ml. of plasma or less alkali may be used for hydrolysis. In normal subjects, 1 ml. or 0.5 ml. plasma may be used. Except in an emergency, using 0.25 ml. of plasma is not recommended since duplicates do not check as well as when larger amounts are used.

Transfer the clot from the tube to the middle of a pile of 3 or 4 pieces of hard filter paper, 9 cm. in diameter, which have been placed on top of a few layers of coarse filter paper or paper towels. Cover with a similar layer of 3 or 4 pieces of hard paper, and put coarse paper on top. Remove water by gently placing a 250 ml. Erlenmeyer flask filled with water on top of the pile (any similar container may be used for pressing out the fluid). In 5 to 10 minutes all fluid will be absorbed by the filter paper, leaving a glistening membrane in the middle of the pile of paper. Loosen the clot carefully from the paper with a needle, stiletto, or fine-pointed glass rod. The membrane can be removed in one piece.

Rinse the clot with 0.9% sodium chloride solution and drop into a pyrex centrifuge tube which has been marked accurately at 5 ml. Add 5 ml. of 1% NaOH and place in a boiling water-bath until clot dissolves (about 15 minutes.) To hasten the hydrolysis, break up the clot by stirring with a glass rod. Cool, remove rod, re-check volume and add water if necessary.

Lowry-phenol-reagent method. Add 0.2 ml. of hydrolyzed fibrin to 10 ml. of Reagent c (working alkaline copper solution) in a test tube or cuvette. At the same time prepare blank and standards (see Table 1). Mix and allow tubes to stand for 15 minutes. Add rapidly 1 ml. of Reagent d (phenol reagent) and mix immediately and thoroughly. Allow all tubes to stand for 30 minutes or longer and read in a photometer at or around 500 mλ. Lowry et al. (4, 5) recommend readings at or near 750 mλ for the range 5-25 γ of protein per ml. of final volume, but for stronger solutions the readings may be kept in a workable range by reading near 500 mλ.

Biuret method. Pipette 2 ml. of hydrolyzed fibrin into test tubes
or cuvettes and add the same quantity of biuret reagent to blank, standards and tests. Read in a photoelectric colorimeter at 500 mµ. See Table 2 for the preparation of standards.

**CALCULATIONS**

\[
\text{Fibrinogen (mg./100 ml. of plasma)} = \frac{\text{Optical Density of unknown}}{\text{Optical Density of standard}} \times \text{mg. protein in standard} \times \left(\frac{100 \text{ ml.}}{\text{ml. plasma aliquot}}\right)
\]

When the clot is formed from 1 ml. of plasma, and 2 ml. of the 5 ml. hydrolyzed fibrin is removed, then the aliquot analyzed is 0.4 ml.—a convenient amount for the biuret method. When 0.2 ml. is removed for the phenol reagent method, then the aliquot analyzed is 0.04 ml. If 0.5 ml. of plasma is employed or the amount of the aliquot varies, appropriate changes must be made in the calculation.

**DISCUSSION**

Since fibrinogen is one of the blood plasma fractions, its separation is fraught with many of the same difficulties found in the analysis of globulins or other components. During the clotting, some of the other protein fractions may be occluded, or there may be incomplete precipitation. No attempt has been made to allow for this (6) since it is known that protein fractions change qualitatively as well as quantitatively during disease processes, and one factor may not hold for all instances. Differences in conditions during the precipitation of the clot and the method of its analysis may account for the variety of normal values found in the literature. It is well for each laboratory to establish its own normal values.

**STANDARDS**

The procedure outlined for the determination of fibrinogen is comparatively simple. The difficulty lies in establishing standards for comparison. It is impossible to obtain fibrinogen commercially of more than 80 to 85% protein. Saifer and Newhouse (6) suggest the use of a partially hydrolyzed fibrinogen standard prepared from normal human plasma, but sometimes this is difficult to obtain and preserve. Any available protein may be used. We found it unnecessary to use fibrinogen as a separate standard since duplicate curves are obtained when fibrinogen, serum albumin, or whole serum are
analyzed within the amounts stated in the phenol method (Table 1) and the biuret method (Table 2).

All standards must be checked by gravimetric or micro-Kjeldahl procedure, and aliquots are run simultaneously with the blanks for each set of plasmas to be tested. For Kjeldahl determination the factor of 6.00 was employed to convert nitrogen into fibrinogen (7) and 6.25 for the albumin and whole serum. Fibrinogen in solution is extremely unstable and must be freshly prepared before use. Solutions of more than 250 mg./100 ml. are made with difficulty. For higher concentration dilute alkali must be added to attain solution.

Morrison (8) has shown that the fibrinogen clot preferentially occludes α- and β-lipoproteins, hence some of the elevated levels in pathologic conditions may be due to lipoproteins rather than the fibrin. The discovery of hypo- or afibrinogenemia as a cause of gross bleeding in parturition has awakened interest in the determination of fibrinogen. Many methods have been proposed that depend on turbidimetric analysis (1, 9), based on salt precipitation. Other "bedside" methods depend upon the disappearance of clotting when thrombin is added to serial plasma dilutions (10). The above methods are rapid and useful in deciding whether the fibrinogen is very low or in the high normal range, but may be equivocal in borderline cases. This is due to many pitfalls in nephelometric analysis (9) under ordinary conditions. The analysis of the isolated clot by chemical means, while less rapid, gives more consistent results.

RESULTS

Fibrinogen was determined simultaneously by both the biuret and the phenol-biuret methods in a series of 50 plasmas in a great variety of conditions (see Table 3). The amount of fibrinogen varied from 110-590 mg./100 ml. The mean value by the biuret method was 349 mg./100 ml. and 347 mg./100 ml. by the Lowry method. Compared statistically, the two methods showed excellent correlation—$t = 2.92; p > 0.01$.

<table>
<thead>
<tr>
<th>Number of plasmas</th>
<th>Biuret method Range mg./100 ml.</th>
<th>Lowry method Range mg./100 ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>110-580</td>
<td>110-590</td>
</tr>
<tr>
<td>Mean value</td>
<td>349</td>
<td>347</td>
</tr>
</tbody>
</table>

$t$ value $= 2.92; p > 0.01$
The fibrinogen values in 20 normal subjects are shown in Table 4. We found a mean value of 295 ± 17.0 mg./100 ml.

**SUMMARY**

A practical method for the determination of fibrinogen is presented in which thrombin is used to convert fibrinogen to fibrin and the resultant clot is hydrolyzed. The concentration of protein may be determined by the usual biuret reagent or by the phenol-biuret reagent of Lowry, if small amounts of fibrinogen are to be measured. The mean normal plasma value was found to be 295 ± 17.0 mg./100 ml.

*Albumin* or *serum* may be used instead of *fibrinogen* as a more convenient protein standard.

**REFERENCES**