An Evaluation of Technics for the Separation and Estimation of Plasma Fibrinogen

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A proposed heat precipitation and thrombin interaction technic for fibrinogen isolation has been compared with a sulfite fractionation technic. All three methods exhibit a high degree of correlation. Fibrinogen, which has been separated from plasma by these technics, may be quantitated by the colorimetric modified biuret and phenol-biuret reaction.

For direct measurement of fibrinogen, heat precipitation followed by ultraviolet spectrophotometry appears to be the technic of choice. The method of measurement at two different wave lengths does not appear to present any advantage over single wave length measurement at 240 and 220 m\text{	extmu}m. Free hemoglobin in concentrations below 800 mg./100 ml. of plasma does not interfere with the sulfite fractionation technic but seriously interferes with the ultraviolet measurements in concentrations above 200 mg./100 ml. Bilirubin interferes with sulfite fractionation in concentrations above 24 mg./100 ml. but not with the heat precipitation technic for fibrinogen estimation. Lipids in excess above normal amounts interfere with both the sulfite fractionation and heat precipitation technics. Normal values for plasma fibrinogen from a variable age group using the sulfite fractionation technic and modified biuret end point have been determined.

Many procedures have been proposed for the determination of fibrinogen. Most of these procedures consist of two steps; namely, the isolation of fibrinogen from other plasma proteins, and its subsequent quantitation by physical or chemical means.

Fibrinogen separation from the plasma proteins may be effected by its interaction with thrombin or calcium to form fibrin, its low coagulation temperature, and its low solubility characteristics in salt mixtures and alcohol. Clotting to form fibrin, by the addition of calcium chloride (1,2,3).
thromboplastin and calcium chloride (3); and thrombin (4-9) to plasma, serves as a basis for most of the isolation technics that have been reported. Fibrinogen may also be separated from plasma by heat precipitation (10-12); whipping out (2); salt fractionation (13-15); ethanol fractionation (16); boundary electrophoresis (17, 18); and zone electrophoresis (19, 20). Other reported technics include interaction of the protein with protamine (21) and antifibrinogen (22, 23).

Technics for the quantitation of fibrinogen include: weighing (2, 7); estimation of nitrogen content (14, 24); colorimetric determination by reaction with phospho-18-molybdic tungstic acid (25); the various modifications of the biuret reaction (9, 15); and the ultraviolet absorption of fibrin dissolved in a 40% (w/v) urea solution (8).

Several turbidimetric methods for the estimation of fibrinogen without prior separation of fibrin have been reported. These direct methods include salting out with ammonium sulfate (26-28), sodium chloride and sodium sulfite (29), sodium sulfate (30); and polymerization of fibrinogen by thromboplastin and calcium (11, 31). Turbidimetric methods are, in general, less sensitive and are subjected to a greater degree of interference from normal and abnormal plasma constituents than methods that require prior isolation of fibrinogen (15, 19).

Electrophoresis, protamine precipitation, and alcohol fractionation technics for isolating fibrinogen from plasma are usually time consuming and unsuitable for routine use. Estimation of fibrinogen with antifibrinogen is limited due to the unavailability of an antiserum in sufficient quantity for routine use and the cost of producing such an antiserum. Fibrinogen values obtained by sulfite fractionation show very good correlation with a thrombin-calcium chloride technic (15). Heat precipitation of fibrinogen as a means of quantitation has not been employed as extensively as conventional clotting technics.

The purposes of this study were to: (1) compare a heat precipitation and thrombin interaction technic for separation of fibrinogen from plasma with a sulfite salt fractionation technic; (2) compare a modified biuret technic for measuring fibrin content with a phenol-biuret reaction and ultraviolet spectrophotometry; (3) determine the effect of added bilirubin, free hemoglobin, and lipids on the sulfite fractionation and heat precipitation technic; and (4) present values for plasma fibrinogen from a variable age group for both males and females.

Methods

Reagents

13% (w/v) Na$_2$SO$_4$ (15)

Benedict's Qualitative Solution (32)
2.5% (w/v) NaOH (carbonate free)

Phenol-biuret protein reagent (Lowry Reagent) (9)

Alkaline carbonate Dissolve 20.0 gm. Na₂CO₃ and 0.5 gm. of potassium tartrate in a liter of 0.1 N NaOH.

Copper sulfate Dissolve 1.0 gm. CuSO₄ . 5H₂O in a liter of distilled water.

Alkaline potassium tartrate copper sulfate solution Mix 45 ml. of alkaline carbonate with 5 ml. of copper sulfate solution. Prepare daily.

0.2 N NaOH for ultraviolet measurements Prepare with water that has been double-distilled from glass.

0.85% (w/v) NaCl Prepare with water that has been double-distilled from glass.

2% (w/v) CaCl₂ solution: Thrombin topical 100 NIH U./0.5 ml. Dissolve the contents of the vial so that 0.5 ml. will contain 200 NIH U. Divide into individual test tubes and freeze.

Stock standard protein solution Dilute 25% normal human serum albumin (salt-poor) to approximately 1.0 gm./100 ml. with 0.85% NaCl solution. Determine the concentration of protein by Kjeldahl nitrogen analysis or by biuret estimation and comparison with a known concentration of albumin standardized by Kjeldahl nitrogen analysis. Adjust concentration with 0.85% NaCl solution to exactly 1 gm./100 ml. Mix well and refrigerate.

Diluted standard solutions of protein Pipet 1.0, 2.0, 4.0, and 8.0 ml. of the stock standard solution of protein into a 10 ml. volumetric flask and dilute to the mark with 0.85% NaCl. Mix well and refrigerate.

Sulfite Fractionation Procedure

In a clean 15 × 120 mm. leak-resistant pyrex screw-cap tube, pipet 1.0 ml. of plasma or 0.5 ml. of plasma followed by 0.5 ml. of 0.85% NaCl solution and mix. Add 9.0 ml. of 13% Na₂SO₃ solution and stand in a 37° water bath for 15 min. Centrifuge at 2500 to 3000 rpm for 5 min. and drain. Wash the precipitate with 5 ml. of 13% Na₂SO₃ by vigorously shaking the tube, while capped. Recentrifuge and allow to drain, being careful not to lose any of the precipitate. Add 5 ml. of 2.5% NaOH solution to the tube and place a cap on it. Place in a boiling water bath and boil until the protein precipitate is dissolved. This is usually required for about 10 min. Cool by immersion in a cold water bath.

Heat Fractionation Procedure

Place 1.0 or 0.5 ml. of plasma into a thick-walled centrifuge tube and mix. Incubate in a water bath at 56–60° for 10 min. Centrifuge at 7900 RCF for 10 min. and drain. Suspend the precipitate in 0.5 ml. of saline
with the aid of a Vortex Mixer. Add 1 ml. of saline, then mix, recentrifuge, and drain. Repeat the last two steps. Dissolve by suspending in 5 ml. of 2.5% NaOH.

**Thrombin Interaction Procedure**

Place 1 ml. of plasma into a tube containing 0.5 ml. of thrombin. Add 4 ml. of 0.85 NaCl and 0.1 ml. of CaCl₂ solution to the tube. Allow to stand at room temperature for 2 hr. Break the clot by vigorous agitation and centrifuge at 3000 rpm for 15 min. Collect the light floating clot by filtration through a gooch crucible, size 3, fitted with Whatman No. 42 filter paper and connected to a suction funnel. Wash the clot with saline. Place both clot and filter paper into a test tube. Add 5 ml. of 2.5% NaOH to the tube. Cap the tube and place in a boiling water bath for 10 min. Cool by immersion in a cold water bath. Centrifuge to remove the undissolved filter paper. Remove the supernatant with the aid of a diluting pipet.

**Modified Biuret Method for Protein**

Add 1.0 ml. of Benedict’s Qualitative Solution to the dissolved fibrin and make up to a volume of 7 ml. with distilled water. Allow to stand 20 min. for color development. Read the color in a spectrophotometer at 555 m<sub>λ</sub> against a blank prepared by adding 5 ml. of 2.5% NaOH to a tube containing 1 ml. of water followed by 1 ml. of Benedict’s solution. A Coleman Jr. Model 6D Spectrophotometer using 19 × 105 mm. cuvettes may be used for measurements.

A calibration curve may be obtained by adding 1.0 ml. aliquots of dilutions of human albumin (representing 100, 200, 400, and 800 mg. of protein per 100 ml. of sample) to 5.0 ml. of 2.5% NaOH followed by 1.0 ml. of Benedict’s Qualitative Solution. Allow to stand for 20 minutes and measure the color formed as outlined above.

**Lowry Method for Protein**

Add 0.4 ml. of dissolved fibrin to 10 ml. of alkaline potassium tartrate copper sulfate solution and allow to stand for 10 min. (At the same time, prepare blanks and standards. This may be suitably done by adding 5 ml. of 2.5% NaOH to 1 ml. of each of the standard solutions and water. Mix and treat 0.4 ml. of the standards and water as outlined above). Add rapidly 1.0 ml. of Lowry phenol-biuret reagent. Mix immediately and thoroughly. Allow all tubes to stand 30 min. and read in a photometer at 700 m<sub>λ</sub>. More concentrated solutions may be read at 500 m<sub>λ</sub>.

**Ultraviolet Method for Protein**

Dissolve the fibrin obtained by heat fractionation in 10 ml. of 0.2 N NaOH for ultraviolet measurements. Dilute 1 ml. of this solution to 10
ml. with 0.85% NaCl. Spectrophotometric measurements may be read against a blank prepared by substituting 1.0 ml. of 0.85% NaCl for the fibrin. Absorbance measurements may be made at wave lengths of 220 m\(\mu\) to 240 m\(\mu\).

**Results**

The optimum temperature for fibrinogen precipitation was determined by incubating pooled plasma specimens at various temperatures ranging from 40° to 80°. No precipitation occurred at 40° and denaturation occurred at 80°. The results of this experiment are shown in Table 1. The amount of protein that precipitated at 56–61° agreed with the values obtained by the sodium sulfite fractionation and thrombin technics.

A comparison of fibrinogen values obtained by sulfite fractionation, thrombin interaction, and heat precipitation using this modified biuret reaction is shown in the scattergram (Fig. 1). Fibrinogen values obtained by the heat precipitation and thrombin technics show a high degree of correlation with the sulfite fraction technic exhibiting correlation coefficients at 0.980 and 0.990, respectively.

The phenol-biuret reaction for protein estimation was compared with

![Graph](image_url)
the modified biuret technic on fibrinogen isolated by sulfite fractionation (Table 2). A regression coefficient of 0.832 and its standard deviation (0.0164) indicates good correlation between the two methods of measurement.

The sulfite fractionation technic did not lend itself to the measurement of protein by ultraviolet spectrophotometry due to absorption by the sulfite ion in this region of the spectrum. However, the heat precipitation technic for separation of fibrinogen proved to be ideally suited for measurement by ultraviolet spectrophotometry. Spectrophotometric analyses were made at 240 and 220 m\(\mu\) and by Waddel’s technic of measuring the difference in absorbance at 225 and 215 m\(\mu\). These results were compared with those obtained by the modified biuret technic. Analyses were performed with a Beckman DU and DB Spectrophotometer. The results of these measurements are shown in Table 3.

As shown, measurements at 220 and 240 m\(\mu\) gave better correlation with the modified biuret method than Waddel’s technic of measuring the difference in absorbance at two wave lengths.

The effect of free hemoglobin on the heat precipitation and sulfite fractionation technics was investigated by adding known concentrations of

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### Table 2. Correlation of 25 Fibrinogen Values Obtained by Sulfite Fractionation*

<table>
<thead>
<tr>
<th>Fibrinogen in plasma (mg./100 ml.)</th>
<th>Biuret method</th>
<th>Phenol-biuret method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Range</td>
<td>250-700</td>
<td>265-680</td>
</tr>
<tr>
<td>Mean</td>
<td>397</td>
<td>392</td>
</tr>
</tbody>
</table>

Regression coefficient \(b\) = 0.832; standard deviation of \(b\) = 0.0164. *Employing a modified biuret and phenol-biuret technic for measuring protein content.

### Table 3. Results on Fibrin Values Obtained by Heat Precipitation*

<table>
<thead>
<tr>
<th>Ultraviolet measurements</th>
</tr>
</thead>
<tbody>
<tr>
<td>240 m(\mu)</td>
</tr>
<tr>
<td>No. tests</td>
</tr>
<tr>
<td>Plasma fibrinogen (mg./100 ml.)</td>
</tr>
<tr>
<td>Range</td>
</tr>
<tr>
<td>Mean</td>
</tr>
<tr>
<td>Regression coefficient (b)</td>
</tr>
<tr>
<td>Standard deviation of (b)</td>
</tr>
<tr>
<td>Coefficient of correlation</td>
</tr>
</tbody>
</table>

free hemoglobin to a pooled plasma specimen and analyzing for fibrinogen. Hemoglobin did not interfere with the sulfite technic in concentrations below 800 mg./100 ml. of plasma. However, ultraviolet measurements of the heat precipitated fibrin showed interference at hemoglobin concentrations above 200 mg./100 ml. This interference is due to incomplete removal of hemoglobin by the technics used for isolation of fibrin. Additional washing appeared to have little effect in removing hemoglobin in concentrations above 400 mg./100 ml.

The effect of added amounts of free bilirubin on recovery of fibrinogen was investigated by adding varying concentrations of bilirubin to pooled plasma. Bilirubin interfered most seriously with the sulfite method. The sulfite tends to salt out the bilirubin, thus rendering it difficult to remove on washing. In concentrations above 24 mg./100 ml., bilirubin interferes with the biuret end point used for measuring fibrin obtained by sulfite fractionation. No interference was noted with ultraviolet spectrophotometric measurement of fibrinogen concentration with plasma containing bilirubin concentrations as high as 120 mg./100 ml. This was due, in part, to the removal of the compound by washing the heat precipitated fibrin with saline.

The effect of lipid concentration on fibrinogen measurement was studied by adding hyperlipemic pooled serum (2790 mg./100 ml. total lipids) to pooled plasma with a lipid concentration of 798 mg./100 ml. Lipid concentration was determined by the method of Bloor (33). The samples were centrifuged at 8000 rpm in an angle-head centrifuge 30 min. prior to being analyzed. The results of this experiment are listed in Table 4. Most of the difficulty encountered was due to turbidity; this interference was partially alleviated by correcting for increased absorbance readings due to turbidity. Absorbance readings of fibrinogen samples were taken

**Table 4. Effect of Lipids on Fibrinogen Values Obtained by Sulfite Fractionation and the Modified Biuret Reagent**

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Total lipids in plasma (mg./100 ml.)</th>
<th>Fibrinogen concentration in plasma (mg./100 ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Sulfite fractionation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Uncorrected</td>
</tr>
<tr>
<td>1</td>
<td>2193</td>
<td>280</td>
</tr>
<tr>
<td>2</td>
<td>1914</td>
<td>290</td>
</tr>
<tr>
<td>3</td>
<td>1635</td>
<td>275</td>
</tr>
<tr>
<td>4</td>
<td>1356</td>
<td>280</td>
</tr>
<tr>
<td>5</td>
<td>1077</td>
<td>265</td>
</tr>
<tr>
<td>6</td>
<td>938</td>
<td>245</td>
</tr>
<tr>
<td>7</td>
<td>798</td>
<td>245</td>
</tr>
</tbody>
</table>

*Using a heat precipitation technic employing ultraviolet spectrophotometric measurements.*
Table 5. Fibrinogen Values of Normal Persons, Obtained by a Sulfite Fractionation Procedure

<table>
<thead>
<tr>
<th>Age group (yr.)</th>
<th>No. of persons</th>
<th>Fibrogen concentration in plasma (mg./100 mL.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Males</td>
<td>Females</td>
</tr>
<tr>
<td>3–19</td>
<td>23</td>
<td>20</td>
</tr>
<tr>
<td>20–39</td>
<td>13</td>
<td>18</td>
</tr>
<tr>
<td>40–59</td>
<td>5</td>
<td>15</td>
</tr>
</tbody>
</table>

at 540 μg before and after the addition of Benedict’s Qualitative Reagent. The readings recorded before the addition of Benedict’s Reagent were multiplied by 0.857 (6/7) to account for volume differences and subtracted from readings obtained after the addition of the reagent. Interference was most pronounced with the heat precipitation technic where measurements were made in the ultraviolet spectral region. The technic of employing the difference in reading between two wave lengths resulted in less interference than quantitating fibrinogen concentration at a single wave length. No pronounced interference occurred with any of the employed technics at lipid concentrations below 1356 mg. Lipid concentrations up to 2000 mg. offered no serious interference with the biuret end point.

Normal values for fibrinogen obtained by sulfite fractionation are listed in Table 5. No significant difference in fibrinogen levels existed between males and females of the same age level. However, there were elevated values in the 3 to 10 age bracket and the 40 to 59 age bracket.

Discussion

The results obtained by the three isolation technics investigated indicate that fibrinogen or fibrin may be quantitatively removed from plasma by both physical and physiological means.

The clot obtained by heat precipitation does not float on the supernatant serum or wash solution in the same manner as clots induced by the action of calcium or thrombin on fibrinogen. In this respect they resemble fibrinogen precipitated by sodium sulfite. Heat precipitated clots are not very soluble in 40% urea solutions (8), thus sharing a property of the fibrin formed by the addition of thrombin or calcium salts to plasma. Fibrinogen precipitated by sodium sulfite is very soluble in 40% urea.

Paper electrophoretic studies were performed on several lots of pooled plasma and fibrinogen precipitated by thrombin, heat, and salt fractionation. In all cases, the fibrinogen was dissolved or partially dissolved, by suspending in 40% urea solution. A purified fibrinogen prepa-
ration* dissolved in urea was studied along with the other preparations. Electrophoresis was conducted in a Durrum cell with a Spinco Model R electrophoresis system for a period of 20–24 hr. Spinco Procedure B was followed (34). In all cases, the protein remained at the point of application and did not migrate with the other protein fractions. When added to plasma or serum, 40% urea did not alter the electrophoretic pattern to any great extent.

Fibrinogen obtained by sulfite fractionation could not be measured by ultraviolet spectrophotometry due to absorption of the sulfite ion. However, the heat precipitation technic for separation of fibrinogen proved to be ideally suited for this purpose. The fibrinogen was thoroughly washed and diluted 1 to 100 prior to measurement, thus reducing the danger of interference by ultraviolet absorbing contaminants.

The heat precipitation technic may be contraindicated for fibrinogen estimation in the presence of pyroglobulins (35). However, this is a very rare occurrence and is usually accompanied by other protein abnormalities. In a series of 20 adult patients who had pyroglobulinemia (36), 10 had myeloma and 1 each had lymphosarcoma, macroglobulinemia, and lupus erythematosus. Two of the patients with myeloma had cryoglobulins present in their serum as well as pyroglobulin. If one of the above conditions is suspected, a sample of serum could be incubated along with the plasma specimen required for fibrinogen estimation. A precipitation in both samples would be indicative of pyroglobulins. The good correlation of fibrinogen values obtained from the randomly selected samples would indicate an extremely low incidence of pyroglobulinemia in the general population.

The addition of the phenol reagent to the hydrolyzed fibrin in the alkaline potassium tartrate copper sulfate solution must be immediate and thorough in the phenol–biuret method for protein estimation. This reagent affords a sensitivity more than 20 times that of conventional biuret technics. Because of the extra precaution required, and a need for scrupulously clean glassware, the reproducibility of this technic is not quite so good as the modified biuret technic. In those cases where sample size is not the limiting factor, the modified biuret technic is preferred for measuring the protein content of fibrinogen. The biuret technic described in this study has twice the sensitivity of conventional biuret technics.

The thrombin isolation technic is the most tedious and time consuming of the three that were investigated. The sulfite fractionation required less time and strict adherence to the details of technic.

*Obtained from Warner-Chilecott, Morris Plains, N. J.
†Beckman Instruments Corp., Spinco Division, Stanford Industrial Park, Palo Alto, Calif.
Although oxalated tubes were used for collecting plasma specimens analyzed in this study, no difference was obtained in fibrinogen values when EDTA, heparin, or citrate were used as anticoagulants for the heat precipitation and sulfate fractionation techniques.

References