Separation and Determination of the Total Globulins of Human Serum

F. Lee Rodkey

A method for isolation and determination of total globulins of human serum, carried out in a single tube without transfer, is described. Serum is treated with 2% (w/v) TCA in ethanol at room temperature to precipitate the globulins quantitatively as a gelatinous white precipitate easily recovered by centrifugation. The supernatant fluid is discarded and the precipitate washed with 2% TCA in ethanol. The globulin precipitate is dissolved in dilute NaOH and biuret reagent is added. There is no interference in the analysis of highly lipemic, icteric, or hemolyzed samples. The method has been applied to a number of abnormal serums, and the reproducibility determined. The globulin precipitation is more reproducible than salt fractionation, and no ether is required for the separation of the albumin from the globulin.

Estimation of globulins in serum is commonly obtained by the difference between the total protein and the albumin content. The biuret reaction as described by Weichselbaum (1) is a convenient and sufficiently sensitive method for estimation of total protein. Various procedures have been developed for the estimation of albumin, which include direct estimation by anion binding (see References 2–6 in preceding paper [2]), and indirect procedures following various fractionation methods. The use of half saturated ammonium sulfate, the classic method for separation of albumin from globulins, is not a convenient routine procedure. Separations with sodium sulfate (3), sodium sulfite (4), and cold methanol (5) have been used. Each of these has serious drawbacks. It is often difficult to obtain a clean, convenient, and reproducible separation of precipitated globulins from the albumin solution. Use of ether to improve

From the Division of Chemistry, U. S. Naval Medical Research Institute, National Naval Medical Center, Bethesda, Md. 20014.
Supported by the Bureau of Medicine and Surgery, Navy Department, Research Task MR005.02-0011.01.
The opinions or assertions contained herein are the private ones of the writer and are not to be construed as official or reflecting the views of the Navy Department or the Naval service at large.
The author is indebted to Dr. Maxime MacKenzie, who performed the electrophoretic analysis.
The human serum and plasma samples used in this study were obtained from the Naval Medical School, National Naval Medical Center, courtesy of Lt. Cdr. Thomas E. Wheeler, (MSC), USN. Received for publication Nov. 9, 1964. Accepted for publication Jan. 11, 1965.
the separation (6) has undoubtedly resulted in many laboratory accidents.

More recently, Delaville et al. (7) and Korner and Debro (8) have shown that albumin is soluble in solutions of trichloroacetic acid (TCA) in ethanol. Others have found that serum albumin is soluble at room temperature in acidified organic solvents such as ethanol, methanol, isopropyl alcohol, and acetone (9, 10). Albumin isolated from serum by such solvents is unchanged with respect to solubility, temperature stability, electrophoretic mobility, dye-binding properties, and immunologic behavior (11–13). There is adequate evidence that albumin isolated from serum is essentially pure. The conditions needed for quantitative separation have not been adequately delineated although Delaville et al. (7) and Debro et al. (14) have described methods for serum analysis based on the solubility of albumin in TCA-ethanol. TCA-isopropanol was used by Watson and Nankiville (10) for separation of albumin, but no data were presented with respect to the quantitative aspects of the fractionation. This report describes the conditions for an essentially quantitative separation of these protein fractions and gives a simple method for estimation of total serum globulins which has a sensitivity similar to that for total protein.

**Materials and Method**

**Reagents and Equipment**

*Trichloroacetic acid, 20% (w/v)* Dissolve 20 gm. of trichloroacetic acid in water and dilute to 100 ml.

*Ethanolic-TCA, 2%* Dilute 5 ml. of 20% TCA with 45 ml. of 95% ethanol. This solution must be freshly prepared daily to avoid interference due to slow formation of ethyl trichloroacetate.

*Sodium hydroxide, 0.2 M* Dissolve 8 gm. of NaOH in 1 L. of water.

*Briet reagent* Stock solution is prepared as given by Weichselbaum (1). Before use, it is diluted with an equal volume of 0.2 M NaOH.

*Protein standard* Clear serum or approximately 6% albumin solution is used. The accurate protein concentration is determined by semimicro-Kjeldahl analysis with proper correction for nonprotein nitrogen.

*Spectrophotometry* Absorbance measurements were made in 1 cm. cells at 555 mµ with a Beckman Model DU spectrophotometer. Other spectrophotometers and cells may be used when the absorbance change with variation in protein concentration has been established.

**Procedure**

**Precipitation of Globulins**

Place 5 ml. of 2% TCA in ethanol in a graduated 13-mm. test tube. Add 0.2 ml. of serum, stopper (a rubber "Vacutainer" stopper is ideal), and
shake vigorously. Allow the tubes to stand at room temperature with intermittent shaking for 2 hr. Centrifuge at about 800 × g for 10 min. to sediment the globulins. Decant and discard the supernatant albumin solution.

Washing of Globulin Precipitate

Add 5 ml. of 2% TCA in ethanol to the precipitated globulins. Stopper the tube, shake vigorously, and immediately centrifuge as before. Decant and discard the wash solution. Drain the inverted tubes over filter paper 10–15 mm. to remove as much of the supernatant fluid as possible.

Biuret Reaction

Add 0.2 M NaOH to the 5.0-ml. mark on the tube, add 2.0 ml. of diluted biuret reagent, and stopper the tube with the same stopper previously used. Dissolve the globulin precipitate by repeated gentle inversion of the tube to insure complete solution of the precipitated globulins. Do not shake this alkaline solution vigorously, as undesirable frothing will occur. Prepare a standard of known protein concentration with 0.2 ml. of serum or albumin standard, 4.8 ml. of 0.2 M NaOH and 2.0 ml. of diluted biuret reagent. Prepare the reference solution, without protein, with 5.0 ml. of 0.2 M NaOH and 2.0 ml. of diluted biuret reagent. Close all tubes with rubber stoppers, and mix the contents by inversion. Incubate the mixtures at 32–35° for 30 min. and determine the absorbance at 555 mμ against the protein-free reference solution set at zero absorbance.

Calculation

Total globulin concentration, G, is calculated from the relation

\[ G = A_0 \times \frac{P}{A_p} \]

where \( A_0 \) is the absorbance of the globulin solution, \( A_p \) is the absorbance of the standard protein solution, and \( P \) is the concentration of protein in the standard.

Results and Discussion

A number of organic solvents, ethanol, methanol, acetone, and isopro-panol, in combination with TCA or HCl were tested for precipitation of globulin at room temperature and at 37°. The use of 2% TCA in ethanol at room temperature as described was the most satisfactory method devised. The same concentration of TCA in isopropyl alcohol or methanol caused precipitation of some albumin as well as globulin. When 0.02 N HCl in methanol was used, the amount of protein precipitated was nearly identical to that precipitated by 2% TCA in ethanol. It was found, however, that slight variation in acid or water content of the methanol caused
appreciable change in the amount of protein precipitated. All combinations of acetone with water and acid which were tested led to precipitation of some albumin as well as globulin.

Delaville et al. (7) have previously shown that precipitation of globulins with TCA-ethanol is time-dependent. The rate of this reaction was therefore studied with a single sample of ACD human plasma at 2 concentrations of TCA. The protein soluble in the supernatant TCA-ethanol solution was determined by semimicro-Kjeldahl analysis at various times after mixing 0.5 ml. plasma plus 5.0 ml. TCA-ethanol. From Fig. 1 it is apparent that after 2 hr. of incubation with either 1 or 2% TCA in ethanol, the residual protein in solution corresponds very closely to the albumin determined by direct analysis of the original plasma with bromocresol green (2). Precipitation periods of less than 1 hr. permit an appreciable amount of globulins to remain in solution.

The fractionation of human serum treated with 2% TCA in ethanol for 2 hr. was also tested by moving-boundary electrophoresis. After removal of the supernate, the precipitated globulins were washed with more TCA-ethanol solution, then dissolved in dilute NaOH. The albumin in the supernatant and wash solutions was precipitated by addition of 2

![Graph](image)

**Fig. 1.** Effect of reaction time and TCA concentration on amount of protein precipitated from human plasma. Fractionation was at 26° with 1% TCA in ethanol (circles) and 2% TCA in ethanol (squares). Albumin concentration of this plasma, measured by spectrophotometric analysis with bromocresol green, is indicated by triangles.
moles of sodium acetate for each mole of TCA, and recovered by centrifugation. The precipitate was redisolved in dilute NaOH. The alkaline solutions of albumin and globulins were then exhaustively dialyzed against barbital buffer pH 8.6, for electrophoresis. The results are shown in Fig. 2 and compared with the electrophoretic diagram of the original serum. Essentially all of the albumin, uncontaminated by globulins, appears with unchanged mobility in the supernate of the TCA-ethanol fractionation. The mobility of the globulins, however, is markedly altered by this procedure to the extent that the isolated globulin fraction moves as a single component with mobility similar to the $\beta$ globulins of the original serum. The very small amount of material (1% of the total protein of the globulin fraction), with mobility similar to albumin, may be either $\alpha$ globulin or albumin. Biuret protein analysis of the samples used for electrophoresis showed that the area under the electrophoretic curve per unit protein nitrogen was identical for the whole serum and the separated albumin and globulin fractions. These data show essentially quantitative separation of albumin from total globulins by this procedure. They also show that the whole serum and the isolated albumin and globulin fractions all have similar reactions with biuret reagent. The total globulins of serum can therefore be measured accurately by this fractionation procedure.

The fractionation procedure was further tested with solutions of crystalline human albumin and human fraction $V^*$ containing about 8 gm. protein per 100 ml. In the case of the crystalline albumin, no globulins

---

*Nutritional Biochemicals Corp., Cleveland, Ohio.
could be detected since no precipitate formed in the 2% TCA-ethanol. A globulin precipitate was formed from the human fraction V preparation which amounted to nearly 3% of the total protein.

Reproducibility of the analysis was tested with a sample of pooled human serum which contained 7.01 gm. total protein per 100 ml. determined by micro-Kjeldahl analysis and corrected for NPN. Fourteen separate analyses were performed by the procedure described. A mean value and standard deviation of 3.83 ± 0.05 gm. of globulins per 100 ml. was obtained. Only 1 measurement differed from the mean value by as much as 0.1 gm./100 ml. Direct spectrophotometric albumin analysis by bromcresol green binding (2) gave a mean value and standard deviation of 3.36 ± 0.05 gm. albumin per 100 ml. for 6 measurements. These data show that the sum of the determined albumin and globulin values is within 2.5% of the total protein concentration of the original serum as determined by Kjeldahl analysis.

A number of serums were analyzed for total globulins by the present procedure and for albumin by the binding of bromcresol green. The results are compared with the total protein analysis in Table 1. These data also show that the sum of the albumin and total globulins as found by the 2 independent methods is in satisfactory agreement with the Kjeldahl value for total protein. In nearly all cases the difference observed was

Table 1. Determined Serum Albumin and Globulin Values and Total Protein Measured by Kjeldahl Analysis

<table>
<thead>
<tr>
<th>Serum</th>
<th>Globulin (gm./100 ml.)</th>
<th>Albumin (gm./100 ml.)</th>
<th>Σ A+G (gm./100 ml.)</th>
<th>TP Kjeldahl (gm./100 ml.)</th>
<th>Deviation of Σ from TP (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.77</td>
<td>4.24</td>
<td>7.01</td>
<td>6.99</td>
<td>+0.3</td>
</tr>
<tr>
<td>2</td>
<td>2.47</td>
<td>4.14</td>
<td>6.61</td>
<td>6.61</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>2.46</td>
<td>4.82</td>
<td>7.28</td>
<td>7.24</td>
<td>+0.6</td>
</tr>
<tr>
<td>4*</td>
<td>3.00</td>
<td>4.94</td>
<td>7.94</td>
<td>7.97</td>
<td>-0.4</td>
</tr>
<tr>
<td>5†</td>
<td>6.30</td>
<td>1.92</td>
<td>8.22</td>
<td>8.18</td>
<td>+0.5</td>
</tr>
<tr>
<td>6</td>
<td>2.44</td>
<td>3.57</td>
<td>6.01</td>
<td>6.09</td>
<td>-1.3</td>
</tr>
<tr>
<td>7</td>
<td>4.98</td>
<td>2.34</td>
<td>7.32</td>
<td>7.13</td>
<td>+2.7</td>
</tr>
<tr>
<td>8</td>
<td>3.80</td>
<td>3.57</td>
<td>7.37</td>
<td>7.26</td>
<td>+1.5</td>
</tr>
<tr>
<td>9</td>
<td>3.06</td>
<td>2.13</td>
<td>5.19</td>
<td>5.44</td>
<td>-4.6</td>
</tr>
<tr>
<td>10</td>
<td>2.65</td>
<td>4.73</td>
<td>7.38</td>
<td>7.08</td>
<td>+4.2</td>
</tr>
<tr>
<td>11</td>
<td>3.58</td>
<td>5.23</td>
<td>8.81</td>
<td>8.49</td>
<td>+3.8</td>
</tr>
<tr>
<td>12</td>
<td>3.42</td>
<td>3.61</td>
<td>7.03</td>
<td>6.65</td>
<td>+5.7</td>
</tr>
<tr>
<td>13†</td>
<td>7.00</td>
<td>1.93</td>
<td>8.93</td>
<td>8.79</td>
<td>+2.2</td>
</tr>
<tr>
<td>14†</td>
<td>4.65</td>
<td>3.97</td>
<td>8.62</td>
<td>8.18</td>
<td>+5.4</td>
</tr>
<tr>
<td>15†</td>
<td>2.74</td>
<td>4.05</td>
<td>6.79</td>
<td>6.61</td>
<td>+2.7</td>
</tr>
</tbody>
</table>

AVERAGE +1.7

* Lipemic.
† High bilirubin.
‡ Hemolyzed.
well under 5% of the Kjeldahl value. Application of the $t$ test for paired observations (15) to these data ($t = 2.55$, 14 degrees of freedom) showed slightly greater than 95% probability that the sum of albumin and globulin analyses is significantly higher than the Kjeldahl value.

The method presented has the following advantages over previous procedures for estimating total globulins in serum: (1) The fractionation procedure, performed at room temperature, is simple, reproducible, and quantitative. (2) The use of ether is avoided. (3) All reactions are carried out in a single, stoppered, graduated tube and no transfer or dilution factors are involved. (4) A single simple protein standard may be used with nearly equal sensitivity for both total protein (1) and globulin determination. (5) Interference in the globulin analysis due to lipemia, hemoglobin, and bilirubin is avoided, since the contaminants in such sera are extracted and discarded with the albumin in the fractionation procedure.

References