Automated Procedure for Simultaneously Measuring Total Globulin and Total Protein in Serum

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An automated continuous-flow procedure has been developed for simultaneously measuring total serum protein and globulin. The method for total protein is a minor modification of an existing automated method in which the biuret reagent is used. Total globulin is measured by reaction with glyoxylic acid, and standardized with N-acetyltryptophan. An empirical factor relating concentration of N-acetyltryptophan to human globulin has been derived. Values for total serum globulin obtained by this new automated procedure correlate closely with values obtained by electrophoresis, but do not agree with values obtained by use of procedures involving binding of anionic dye. Recovery of gamma globulin added to serum is essentially quantitative; the day-to-day precision (CV) is 4.31%.

Additional Keyphrases  N-acetyltryptophan as a reference material • comparison with dye-binding and other procedures • glyoxylic acid reaction • normal values • AutoAnalyzer • biuret reaction

In most methods for measuring total serum proteins the biuret reagent (1–4) is used, and this reaction has been successfully automated in a continuous-flow system (5).

Albumin:globulin ratios have usually been estimated by salt fractionation (1–4, 6, 7) or electrophoretic (8, 9) techniques. However, such procedures are not readily adaptable to automation. Albumin binds selectively to some anionic dyes, and spectrophotometry or fluorometry has been used to measure the resulting complex. The anionic dye most commonly used for this purpose has been 2-(4'-hydroxybenzeneazo)-benzoic acid (HABA) (10–12), although methyl orange (5, 13–15) and bromocresol green (16–18) have also been used. There is some disagreement, however, as to the reliability of the dye-binding methods; falsely increased albumin values are obtained because dye may be bound to proteins other than albumin.

Recent reports (19–20) describe a direct spectrophotometric procedure for the measurement of total serum globulin, in which serum is added directly to a reagent containing glyoxylic acid in an acetic acid solution.

The glyoxylic acid reaction has been used by several investigators (21–26) to determine tryptophan-containing proteins such as serum globulins, which contain 2.0 to 3.0% tryptophan. The determination depends on the purple color formed when glyoxylic acid reacts in a strongly acid medium with tryptophan or tryptophan-containing compounds. The color is more intense if cupric ions are present in the reagent. Hopkins and Cole (21), in 1901, first described the glyoxylic acid reaction with tryptophan, which was subsequently developed as an analytical method by Fischl (23) and Shaw and McParlane (22).

Here, we have adapted the direct spectrophotometric procedure employing the glyoxylic acid reaction for the measurement of total serum globulin to an automated continuous-flow system. Simultaneous measurement of total protein by the well-established biuret method is also described.

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Materials and Methods

Apparatus

**Automated system.** A manifold and flow system assembled for use with modules of the Auto-Analyzer (Technicon Instruments Corp., Tarrytown, N.Y. 10591) are shown in Figure 1.

**Electrophoresis.** The Microzone System (Beckman Instruments, Inc., Spinco Division, Palo Alto, Calif. 94304) is used for serum protein electrophoresis, and electrophoretograms are scanned on the Beckman Microzone Densitometer, Model R-110.

Reagents

**Glyoxylic acid reagent.** This reagent consists of an aqueous solution containing 800 mg of glyoxylic acid, 600 mg of copper sulfate, 15 mol of acetic acid, and 2.2 mol of sulfuric acid per liter. This preparation may be obtained commercially ("Diagnostest," Dow Chemical Co., Indianapolis, Ind. 46206).

**N-acetyl-DL-tryptophan stock standard, 4.4 g/liter (equivalent to 80 g of globulin per liter).** To 4.40 g of vacuum-dried N-acetyl-DL-tryptophan ("purified" grade, Sigma Chemical Co., St. Louis, Mo. 63118) add about 200 ml of distilled water followed by 25 ml of 0.75N sodium hydroxide and mix until dissolved. Dilute to 1 liter with distilled water. Dispense this standard solution into 4-ml aliquots and freeze.

The purity of the N-acetyl-DL-tryptophan was established by elemental analysis of the dried commercial compound. Calculated for C_{13}H_{14}N_{2}O_{4}: C, 63.38; H, 5.73; N, 11.38. Found: C, 62.38; H, 5.67; N, 11.32.

**N-acetyl-DL-tryptophan working standards.** Appropriate dilutions of stock standard are made daily to give working standards of 55, 110, 220, and 330 mg/100 ml (equivalent to 10, 20, 40, and 60 g of globulin per liter).

**Total protein reagents.** The biuret reagent is
prepared as described by Weischelbaum (27). The blank reagent consists of 5 g of potassium iodide and 8 g of sodium hydroxide diluted to 1 liter with distilled water.

**Protein stock standard.** One hundred grams of bovine albumin per liter, fraction V (Technicon) is dissolved in NaCl solution, 9 g/liter. The supplier of the bovine albumin lists its total nitrogen content by Kjeldahl analysis and we correct for non-protein impurities by using a 6.54 nitrogen factor (28).

**Working protein standards.** The stock standard is diluted to achieve concentrations of 20, 40, 60, 80, and 100 g/liter.

**Human albumin standards.** Prepared from human mercaptoalbumin (Mann Research Laboratories, New York, N.Y. 10006) as described for the bovine albumin standards.

Methods

**Automated total protein and globulin.** At a sample rate of 40/h and with a 1:1 sample to wash ratio, specimens are initially diluted in saline, mixed, and subsequently separated into the individual channels where the biuret and glyoxylic acid reactions are carried out.

In the estimation of total globulin, samples are mixed with the glyoxylic acid reagent and color is developed in a 25-m time-delay coil contained in a temperature-controlled heating bath at 95°C. The resulting violet color is measured in a colorimeter at 550 nm.

Total protein is measured by essentially the method of Failing et al. (5), adapted for the present continuous-flow system with a few minor modifications. The standards for total protein and total globulin are inserted at the beginning and completion of the daily run, in order of increasing concentrations. Combined total protein and globulin standards are not prepared, since the bovine albumin used for the total protein standard gives some color with the glyoxylic acid reagent.

**Serum protein electrophoresis.** The Beckman “Microzone” system is used as described by Kaplan and Savory (29).

**Albumin determined by anionic-dye binding.** The automated procedure using HBABA dye is used as described by Nishi and Rhodes (12).

**Albumin and globulin via salt fractionation.** The method of Wolfson et al. is used (4).

Results and Discussion

Total Protein Measurements

Total protein was measured by a modification of the automated procedure described by Failing et al. (5) by use of the Weischelbaum formula (27) for the biuret reagent. The modification consisted of initial mixing of the serum with saline in the continuous-flow system before total protein and globulin were simultaneously determined. This initial dilution was important for the globulin measurements and also ensured that absorbances would fall in the desired 0.2 to 0.4 range for the total protein determinations. Blanks were measured for moderately hemolyzed, lactescant, and icteric sera by using the blank reagent in place of the biuret reagent.

To test the validity of the new modified method, we analyzed 40 samples both by this procedure and by Failing’s procedure. The slope of the regression line was 1.008 with a standard error of estimate of 0.9 g/liter and a correlation coefficient of 0.995. Thus, a very close correlation exists between results obtained by the two methods.

Total Globulin Measurements

A recorder tracing from the continuous-flow procedure for serum globulin is shown in Figure 2. At the sampling rate described in the Methods section, only slight interaction of samples was observed. No carryover was detected when the 20 or 40 g/liter standards followed the 60 g/liter standard, and only 5% carryover was observed due to interactions of the 60 and 10 g/liter standards.

**Color development.** The incubation time required for maximum color development of glyoxylic acid–globulin reaction was investigated by collecting a sample of diluted serum mixed with the glyoxylic acid reagent at the H3 connector.
(Figure 1) during continuous aspiration of a normal serum sample, and heating at 95°C for 1 to 8 min. The absorbances of the reaction mixtures were measured in 12-mm cuvets, and maximum color developed after 7 min. In the present automated procedure, serum and reagent are present in the time-delay coil at 95°C for 4 min, which corresponds to 60% of maximum color development.

**Standardization.** Goldberg and Drews (19) reported that N-acetyltryptophan reacted with the glyoxylic acid reagent and could be used to standardize the manual serum globulin procedure. Their approach to standardization was followed in the present procedure. N-acetyltryptophan can be purchased in a pure form and is quite soluble in dilute alkaline solution, thus fulfilling the requirements for a primary reference material.

To relate concentration of N-acetyltryptophan to total globulin concentration, we determined an empirical conversion factor. This factor was derived by obtaining 28 serum samples from normal individuals and measuring their total globulin concentration by electrophoresis on cellulose acetate. These values were confirmed by the salt fractionation procedure of Wolfson et al. (4), which we find correlates closely with the results obtained by electrophoresis. The same samples were analyzed by the automated globulin method and the peak heights related to a standard curve prepared by use of N-acetyltryptophan. By calculating the means of the 28 samples, we established that 550 mg of N-acetyltryptophan per liter is equivalent to 10 g of total globulin in serum per liter in terms of intensity of color developed with the glyoxylic acid reagent. This factor corresponds to a tryptophan content of the globulin of 4.55%, a value well above the reported tryptophan content of 2.0 to 3.0% (30). This deviation is mainly caused by the contribution of albumin, which contains 0.2% tryptophan and constitutes about 60% of serum proteins in normal individuals. In addition any derivative of tryptophan containing the indole ring produces color equivalent to that produced by tryptophan with the glyoxylic acid reagent (23), and any compounds of this type present in serum will contribute color.

Essentially, the present method of standardization with N-acetyltryptophan has employed sera of known albumin:globulin ratios as primary references, which is the procedure used by Saifer et al. (24). However, because of the difficulties in the day-to-day preparation of solutions of various globulin concentrations, we find N-acetyltryptophan to be more satisfactory. N-acetyltryptophan is acceptable as a reference material because it undergoes the same reaction with the glyoxylic acid reagent as do the serum globulins. N-acetyltryptophan can be obtained in a pure state and, for day-to-day standardization, should be definitely superior to reference materials prepared from commercially obtained protein fractions whose purity and tryptophan content might vary. Any laboratory can obtain (or prepare) N-acetyltryptophan of the same purity as that described in the present paper and, therefore, can obtain results for standards that are identical to ours.

The stability of the N-acetyltryptophan working standards, when stored at −10°C, was determined at weekly intervals by comparing results with those for freshly prepared standards. No deterioration was observed during 18 weeks.

An alternative standardization procedure, in which human gamma globulin dissolved in saline solution (9 g/liter) was used, was also considered, but we saw no advantages over the N-acetyltryptophan. Problems were encountered with gamma globulin as a standard: it was difficult to prepare sufficiently high globulin concentrations that were stable and did not precipitate when stored at −10°C.

The present continuous-flow automated system gave a nonlinear standard curve with the N-acetyltryptophan and with standard solutions of human gamma globulin in saline solution (9 g/liter). Both curves could be made linear by mixing serum directly with glyoxylic acid reagent, thus eliminating the initial dilution of serum with saline. The only difference between the two methods was the amount of water in the final reaction mixture. The increased amount of water in the present automated method apparently resulted in the nonlinear standard curve. If initial dilution of saline and sample was omitted in the reaction, a flow rate of 7.8 ml of glyoxylic acid reagent per minute was required with a sample pickup rate of 0.05 ml/min to achieve absorbances in the 0.1 to 0.6 range. When a sample line of such small interior diameter was used, problems of clogging were encountered. In our opinion, the nonlinearity of the present system presents no problems since, as with most automated methods, several standards are run with each set of samples.

**Interferences.** Several compounds present in serum were tested to see if they would interfere with the automated measurement of serum globulins. Solutions of urea (500 mg/liter), glucose (2.5 g/liter), creatinine (100 mg/liter), and uric acid (120 mg/liter) were put through the automated procedure; none produced any considerable color with the glyoxylic acid reagent.

Lipemic and hemolyzed sera contributed no appreciable color at 550 nm, as determined by running blanks on such sera, and concentrations of bilirubin in the range of 150 mg/liter reacted similarly. Saifer et al. (24, 26) experienced some interference from sera from jaundiced patients, but their method involved a 1:50 dilution of serum,
Table 1. Recovery of Bovine Gamma-Globulin Added to Serum

<table>
<thead>
<tr>
<th>No. samples</th>
<th>γ-Globulin added, g/liter</th>
<th>γ-Globulin recovery, g/liter</th>
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<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
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<tr>
<td>10</td>
<td>12</td>
<td>±0.4</td>
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<td></td>
<td>(100%)</td>
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</tr>
<tr>
<td>10</td>
<td>17</td>
<td>±0.4</td>
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<tr>
<td></td>
<td>(94%)</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>37</td>
<td>±1.4</td>
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<tr>
<td></td>
<td>(97%)</td>
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as compared with the 1:230 dilution in our automated procedure. The increased dilution obviously would decrease the proportionate contribution of bilirubin to the final color.

Some color was produced from human albumin, as expected, since this protein contains 0.2% tryptophan (30). The absorbances produced by human mercaptalbumin solutions of 20 to 100 g/liter were 16% as large as those produced by globulin solutions of equivalent concentrations. However, the error produced by this albumin interference was minimized by the standardization process used in the automated method. As discussed previously, standardization was accomplished by using 28 normal sera for which the globulin content had been determined by electrophoresis and related to the color produced with the glyoxylic acid reagent by these sera and by known concentrations of N-acetyltryptophan. Each of these 28 sera contained normal concentrations of albumin and therefore the factor relating globulin and N-acetyltryptophan concentrations takes into account the color produced with glyoxylic acid by the albumin present.

Some error is, of course, introduced when the factor obtained for normal sera is applied to abnormal sera. However, Saifer et al. (24) pointed out that the tryptophan content of normal serum (40 g of albumin per liter) and that of abnormal serum (20 g of albumin per liter) differs negligibly when one considers that albumin contains only 0.2% tryptophan, compared to the 3% present in the human globulin fraction. The results of our study bear out this observation for the present automated method and those that use electrophoresis or salt fractionation in the case of the many samples with abnormal albumin concentrations that we examined.

Precision. The precision of the automated globulin method was determined from the results of 70 daily measurements of a pooled serum sample. The mean was 26.3 g/liter with a standard deviation of ±1.1 g/liter and a coefficient of variation of 4.31%.

Recovery tests. Globulin recovery was measured by adding to 10 sera a solution containing 92 g of bovine gamma globulin per liter, dissolved in saline, 9 g/liter. The recoveries (Table 1) are essentially quantitative for each amount of globulin added.

Comparison with other methods. Total globulin concentrations of 220 sera from hospital patients were determined by both the automated glyoxylic acid and cellulose acetate electrophoresis procedures, the latter being a reference method for serum protein fractionation. Of the 220 samples, 91 had normal electrophoretograms, 30 showed hypoalbuminemia with polyclonal hyperglobulinemia, 11 showed hypoalbuminemia with hyperalpha-2-globulinemia, one demonstrated a monoclonal spike in the gamma region, and the remainder had various abnormal patterns. Several specimens were also icteric, hemolyzed, or lipemic. The correlation of the two methods is given in Figure 3. The correlation coefficient for these data was 0.915 with a standard error of estimate of 3.0 g/liter. In contrast, the correlation of globulin values obtained from these 220 samples by using the automated glyoxylic acid and HBABA dye procedures showed a slope of the regression line of 0.889 with a standard error of estimate of 5.5 g/liter and a correlation coefficient of 0.800. Thus, the HBABA dye method provided significantly lower results for total serum globulin than either the automated glyoxylic acid or electrophoresis procedures. Furthermore, none of the 220 samples gave any appreciable blank in the automated method when the saline solution was substituted for the glyoxylic acid reagents despite the fact that several of the specimens were moderately to markedly hemolyzed, icteric, or lipemic. In contrast, the HBABA dye procedure gave considerable blanks with many specimen,
and a blank correction on all tests was found to be necessary. These results clearly indicate the advantages of this new automated system over the widely used automated HBABA dye method for measuring serum protein fractions.

References


