Albumin Quantitation by Dye Binding and Salt Fractionation Techniques

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Two dye-binding reagents, 2-(4'-hydroxybenzeneazo)benzoic acid (HABA) and brom cresol green (BCG), for the quantitation of serum albumin have been compared with the biuret colorimetric procedure after salt fractionation. The linear regression equations for the biuret-BCG and the biuret-HABA comparisons were \( y = 0.43 + 0.91x \) and \( y = -0.45 + 1.15x \), respectively. Their corresponding correlation coefficients were 0.93 and 0.83, respectively. The effects of icterus, lipemia, and hemolysis on the dye-binding procedures were generally greater in the case of the HABA procedure. A comparison of results with BCG and HABA for 20 sera having A/G ratios of less than 1, indicates them to be equally reliable.

Additional Keyphrases: HABA method, brom cresol green method, effects of icterus, lipemia, hemolysis

Serum albumin quantitation based upon the "protein-error" of indicators was introduced in 1953 by Bracken and Klotz (1). Since then, there has been an increasing number of dye methods for the determination of serum albumin, with various attendant modifications. The dyes in widest use have been the azo dyes such as methyl orange and 2-(4'-hydroxybenzeneazo)benzoic acid (HABA). More recently phthalene dyes such as brom cresol purple (2) and brom cresol green (BCG) (3-10) have been used for this purpose.

The BCG method, initiated by Rodkey (3, 4), has undergone modifications, primarily a change of reagent pH from 7.0 to 4.0 and the inclusion of a nonionic surfactant. These changes have resulted in absorbance readings that are directly proportional to albumin concentration and reagent blanks with relatively low absorbances.

Here, we compare the merits of the BCG and HABA methods with the biuret colorimetric technique after salt fractionation. The BCG and HABA methods were compared in the analysis of sera with normal and abnormal albumin/globulin ratios. In addition the effects of icteric, lipemic, and hemolyzed serum specimens on the dye-binding methods were studied.

Materials and Methods

For all methods a commercial control serum was used as the reference base ("Versatol," lot No. 0814108; General Diagnostics Division, Warner-Lambert Pharmaceutical Co., Morris Plains, N. J. 07950).

Brom cresol Green (BCG) Method (11)

Stock reagent consisted of 26 ml of sodium hydroxide (100 g/liter), 30 ml of lactic acid, 500 mg of BCG, and 10 ml of "Tween 20," all diluted to 1 liter with distilled water, mixed, and the pH adjusted to 4.0 at room temperature ("AlbuStrate," General Diagnostics).

The stock reagent was diluted five-fold with distilled water before use. Ten microliters of serum was added to 5 ml of diluted reagent, mixed, and read at 630 nm vs. the reagent blank.

HABA Dye Method

The procedure was performed on the Model DSA 560 (Beckman Instruments, Fullerton, Calif. 92634) according to Beckman Procedure No. 83917, a modification of the method described by Martinsek (12). In the modified HABA method, HABA is used at a concentration of 1.5 mmol/liter in a phosphate buffer of pH 6.4, the detergent, "Brij-35," is incorporated and the instrument is usually set on a serum blank. Serum blanks were prepared by substituting HABA reagent with phosphate buffer. All absorbances were read at 510 nm. The manual HABA method was identical to the automated procedure except for a proportional increase in reagent volumes; where HABA reagent blanks were used, serum was replaced by water.

Biuret Colorimetric Method

After Salt Fractionation

The salt fractionation technique was performed according to the method of Gornall et al. (15). A sodium sulfate-sulfite mixture (270 g/liter) was used for fractionation of the albumin and globulins.
Methods of Investigation

The three methods were first compared by analysis of 120 random sera from hospital patients. All analyses were carried out within a 24-h period from the time of collection. Icteric, hemolytic, or lipemic sera were noted. Twenty sera having a normal appearance but abnormal albumin/globulin ratios were analyzed for albumin by the three procedures under study.

The effects of bilirubin and hemoglobin on serum albumin determinations were investigated by use of the following solutions:

**Bilirubin.** Bilirubin solutions were prepared in 20 µg/100 ml of sodium carbonate in concentrations ranging to 50 mg/100 ml (bilirubin; Harleco, Philadelphia, Pa. 19143). Vials of lyophilized control serum were reconstituted with 5 ml of the above bilirubin solutions before analysis.

**Hemoglobin.** A concentrated hemoglobin solution was freshly prepared by hemolyzing washed red cells with water. The hemolysate was analyzed and appropriately diluted with saline to give concentrations ranging up to 500 mg/100 ml. Vials of lyophilized control serum were reconstituted with 5 ml of the above hemoglobin solutions before analysis.

The effects of bilirubin and hemoglobin on the BCG and HABA dye methods were further investigated by using an aqueous solution of purified human serum albumin (Human 4X recryst. serum albumin; Nutritional Biochemical Corp., Cleveland, Ohio 44128). As described above, bilirubin and hemoglobin solutions were prepared and added in appropriate amounts to aqueous solutions of purified serum albumin. The resulting solutions were analyzed by both BCG and HABA methods; however, in the latter series of experiments, spectral absorbance patterns were obtained instead of absorbance measurements at a fixed wavelength. All spectral patterns were obtained with a Bausch & Lomb Spectrophotometer, Model 600, coupled to a Sargent SRL Recorder.

Results and Discussion

Spectral absorbance curves for the BCG and HABA methods are illustrated in Figure 1. Note that whereas BCG measurements made at 630 nm correspond to its spectral absorbance maximum, the wavelength of 510 nm used for the HABA method does not correspond to its blank-corrected spectral absorbance maximum, which is about 485 nm. Measurements at 510 nm are less sensitive because of the lower absorptivity. Comparative sensitivities of the BCG, HABA and biuret methods are shown in Table 1. The BCG method is perhaps 20 times as sensitive as the HABA method.

![Spectral absorbance curves](image)

**Fig. 1.** Spectral absorbance curves obtained on an albumin solution by HABA and BCG methods

**Composition of solutions used:**

<table>
<thead>
<tr>
<th>Designation</th>
<th>Albumin, g/100 ml</th>
<th>Dye</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>4</td>
<td>BCG</td>
<td>Water</td>
</tr>
<tr>
<td>B</td>
<td>0</td>
<td>BCG</td>
<td>Water</td>
</tr>
<tr>
<td>C</td>
<td>4</td>
<td>HABA</td>
<td>Water</td>
</tr>
<tr>
<td>D</td>
<td>0</td>
<td>HABA</td>
<td>Water</td>
</tr>
</tbody>
</table>

**Table 1. Comparative Data for Albumin Determinations by HABA, BCG, and Biuret Methods, with Purified Human Serum Albumin**

| Method   | Sample size, ml | Total volume reagent sample, ml | Sample to total volume ratio | Cuvet size, mm | Absorbance | Sensitivity$
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>HABA</td>
<td>0.050</td>
<td>3.80</td>
<td>76</td>
<td>10</td>
<td>0.15</td>
<td>11.4</td>
</tr>
<tr>
<td>Biuret</td>
<td>0.200</td>
<td>20.00</td>
<td>100</td>
<td>19</td>
<td>0.14</td>
<td>0.7</td>
</tr>
<tr>
<td>Albustrate</td>
<td>0.010</td>
<td>5.01</td>
<td>501</td>
<td>10</td>
<td>0.44</td>
<td>220</td>
</tr>
</tbody>
</table>

* All absorbance measurements were read on a Coleman Jr. Spectrophotometer, Model C. The albumin analyzed was 4 X crystallized human serum albumin prepared at a concentration of 4 g/100 ml. All samples were read against appropriate reagent blanks.

$^1$ Sensitivity = \( \frac{(A)\text{(sample : vol. ratio)}}{d} \), where A is absorbance and d is cuvet size.
Comparisons of the BCG and HABA methods with the biuret method were made with scattergram plots (Figures 2 and 3). Both visually and statistically, BCG measurements correlate better than the corresponding HABA measurements with those obtained by biuret after salt fractionation; this is especially true in cases of icteric and lipemic sera, but this difference is negligible in the case of hemolytic sera.

The effect of lipemia was found to be greater with the HABA procedure. An explanation for this difference can probably be based on the greater sensitivity of the BCG procedure, which requires less serum and therefore less lipemia-causing lipids.

The quantitative effects of bilirubin and hemoglobin on the BCG and the HABA methods for the determination of albumin were investigated (Table 2). With the HABA method, the results indicate increased absorbances with increased concentrations for both bilirubin and hemoglobin when uncorrected with a serum blank. This increase amounts to approximately 1% per milligram of bilirubin and an 18% increase in absorbance at a hemolglobin concentration of 500 mg/100 ml. If serum blank-corrected absorbance readings (absorbance differences) are compared instead of total absorbance readings, then the albumin values are found to be decreased with icteric sera. This apparent depression of albumin values, amounting to approximately 0.4% per milligram of bilirubin, can probably be ascribed to an “over-correction” imposed by reading the test against a serum blank (see Table 2 and Figure 4). With hemoglobin, a serum blank adequately corrects for added hemoglobin. In both cases, the blank-corrected results are more acceptable than the corresponding non-corrected ones.

On the other hand, with the BCG method, bilirubin and hemoglobin interferences were minimal, amounting to 0.04% per milligram of bilirubin and 3% at a hemoglobin concentration of 500 mg/100 ml.

The effects of bilirubin and hemoglobin on BCG and HABA methods were further investigated with use of purified serum albumin (Figures 4, 5, and 6). The spectral absorbance curves obtained with the HABA method indicate a shift in the maxima to lower wavelengths with increased bilirubin concentration for both test and serum blank series. This effect is more marked with the test series and can probably be explained, in part, as the additive superimposition of the spectral patterns for HABA-albumin and bilirubin. A comparison of the series of curves obtained with icteric sera indicates a small but significant difference in the spread of the absorbance measurements at 510 nm between the serum blank and test series. The greater spread in the absorbances of the serum blank series with

Table 2. Effect of Bilirubin and Hemoglobin on the HABA and BCG Dye Methods for the Determination of Serum Albumin

<table>
<thead>
<tr>
<th>Concentration mg/100 ml</th>
<th>Absorbancesa</th>
<th>HABA</th>
<th>Test</th>
<th>Serum blank</th>
<th>BCG</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bilirubin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.016</td>
<td>0.010</td>
<td>0.150</td>
<td>0.440</td>
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<tr>
<td>10</td>
<td>0.170</td>
<td>0.025</td>
<td>0.155</td>
<td>0.445</td>
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</tr>
<tr>
<td>20</td>
<td>0.185</td>
<td>0.045</td>
<td>0.140</td>
<td>0.450</td>
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<td></td>
</tr>
<tr>
<td>30</td>
<td>0.205</td>
<td>0.062</td>
<td>0.143</td>
<td>0.450</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>0.230</td>
<td>0.100</td>
<td>0.130</td>
<td>0.450</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemoglobin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.160</td>
<td>0.010</td>
<td>0.150</td>
<td>0.440</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>0.165</td>
<td>0.015</td>
<td>0.150</td>
<td>0.440</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>0.165</td>
<td>0.016</td>
<td>0.149</td>
<td>0.434</td>
<td></td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>0.174</td>
<td>0.020</td>
<td>0.154</td>
<td>0.449</td>
<td></td>
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</tr>
<tr>
<td>300</td>
<td>0.185</td>
<td>0.022</td>
<td>0.163</td>
<td>0.449</td>
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<td></td>
</tr>
<tr>
<td>500</td>
<td>0.188</td>
<td>0.034</td>
<td>0.154</td>
<td>0.454</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Concentration of bilirubin and hemoglobin added to a commercial, lyophilized control sera for which the bilirubin concentration was 0.8 mg/100 ml and hemoglobin was undetectable by the Porter method (15).

† Absorbance readings obtained on the Coleman Jr. Spectrophotometer, Model C, estimated to the third place.
icteric sera would "overcorrect" for the bilirubin present, and the results confirm previous findings (Table 2).

The difficulty encountered with the HABA method for icteric sera is made clear for those utilizing this procedure on some of the more popular instruments for automated analysis. Almost invariably, when an albumin result by the HABA method performed on an icteric serum is suspected of being falsely low, and is compared with that obtained by the BCG method, the result by the latter method will be found to be substantially higher. These differences have not been found to be directly related to the bilirubin concentration of serum and, as shown by the investigations of Arvan and Ritz (14), the apparent depression of albumin values obtained by the HABA method could be related to the conjugated fraction of bilirubin—free bilirubin had little effect. Their findings are not inconsistent with ours and offer a logical explanation for the inconsistency in albumin values by the HABA method in icteric sera. A more definitive study will require a better knowledge of the true nature of the chemical entities involved and of their interactions, information not now clear.

The effect of hemoglobin on the HABA method is shown in Figures 5 and 6. The results are not particularly remarkable; there is no shift in the absorbance maximum with increased hemoglobin concentrations up to 500 mg/100 ml, there appears to be little interference from the 540 nm peak of oxyhemoglobin, and the difference in the spread of absorbance at 510 nm for the blank and the test series appears insignificant. The last observation confirms the findings of Table 2, and indicates that the HABA method with serum blank correction yields results that are uninfluenced by hemoglobin.

Two properties of the BCG method stand out: (a) the greater sensitivity and specificity of BCG for albumin quantitation minimize interference by other foreign serum chromogens, and (b) the greater difference in the wavelength of measurement for the BCG method (630 nm) in comparison to that for the HABA method (510 nm) minimizes interference from substances such as bilirubin (460 nm) and oxyhemoglobin (418, 542, and 582 nm).

The BCG and HABA methods for determination of serum albumin were compared for 20 sera having A/G ratios of less than 1 (Table 3). The 20 samples were considered to have an abnormal A/G ratio
and were selected because of no demonstrable evidence of lipemia, hemolysis, or icterus. The two methods measure albumin equally reliably. In view of these results and findings of an occasional unreliable albumin result by the HABA procedure, for sera with abnormal A/G ratios, it appears that these discrepancies might result from the analysis of icteric, lipemic, or hemolytic (uncorrected for serum blank) sera. Or possibly there might be interference from compounds that competitively bind albumin such as fatty acids, sulfonamides, salicylates, glucuronides, and phenobarbital. We are currently investigating the effect of these compounds on both the HABA and the BCG methods.

References
11. Babson, A. L., see Addendum to this paper.

Addendum

The bromcresol green albumin procedure described in the preceding publication was developed in our laboratories and submitted to CLINICAL CHEMISTRY for publication. In the interim an essentially identical procedure was published by Doumas et al. (1). These authors used 105 mg of bromcresol green per liter of succinate buffer (75 mmol/liter, pH 4.2) containing "Brij-35" (1.2 ml/liter), while our assay uses 100 mg of bromcresol green per liter of lactate buffer (0.1 mol/liter, pH 4.0) containing 2 ml of "Tween-20." Doumas et al. also used 25 ml of serum in 4 ml of reagent, while our method calls for 10 ml of serum in 5 ml of reagent. We have compared the two methods, and they give similar results.

The advantages of an acid-buffered solution of bromcresol green containing a nonionic surfactant as a reagent for serum albumin were thoroughly documented by Doumas et al. We could see no justification for burdening the literature with substantially the same information, and reluctantly withdrew our paper from consideration by CLINICAL CHEMISTRY.

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