Determination of Sulfobromophthalein in Serum

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Since the introduction of sulfobromophthalein (BSP) as a test for liver function, (1) it has gained wide acceptance and is considered (2) the most reliable single test for evaluating liver function. BSP extraction by the liver has also been used as a measure of hepatic blood flow.

Measurement of BSP in water solution is relatively simple in that the compound is a dye, purple in alkali, and colorless in acid. The difference in intensity of color when pH is changed from alkaline to acid is proportional to concentration. In serum, the presence of turbidity, bilirubin, or hemoglobin interferes with the analysis because these factors change absorbance with sharp changes of pH and are falsely read as BSP. Gaebler (3) made corrections for these factors by reading optical densities at 580 and 620 mμ and applying a correction. Reinhold (4) made readings at 420, 580, and 660 mμ to correct for hemolysis, jaundice, and turbidity. The method to be described reduces the number of colorimetric readings to a single wavelength. It eliminates interference from the above factors by minimizing the pH change to the minimum required for the color measurement of BSP. Furthermore, the effect of protein-binding of BSP is eliminated by adding a large excess of anions to the reaction mixture.

Apparatus

1. Photometer. For analytical work, measurements were made in a Bausch and Lomb monochromatic colorimeter using a 575 mμ inter-
ference filter and an 18 mm. test tube cuvet. The latter requires a minimum of 4.0 ml. of colored solution. Adaptations to smaller or larger cuvets or other photometers can be made readily. Spectral-absorbance curves were obtained on the Beckman DU Spectrophotometer.

REAGENTS

1. **BSP standard, 10.0 mg. per 100 ml.** This solution is equivalent to the 100 per cent retention standard for the 5 mg. per kilo test dose of BSP (5). Pure BSP can be obtained from Hynson, Westcott and Dunning in Baltimore as "Bromsulphalein." Their test solution which is readily available, contains 50.0 mg. per ml. It is diluted 1 to 500 to make the 10.0 mg. per 100 ml. standard. (A personal communication from Dr. John Brewer indicates that this test solution varies from 98 to 102 per cent of the stated value and is, therefore suitable for standardization of the BSP method).

2. **Alkaline buffer pH 10.6-10.7.** Dissolve 12.2 Gm. of Na₂HPO₄·7H₂O, 1.77 Gm. Na₆PO₄·12H₂O and 3.20 Gm. sodium p-toluenesulfonate and make to 500 ml. with water. Adjust to pH 10.6 to 10.7 with 1 N NaOH or 1 N HCl.

3. **Acid reagent, 2M NaH₂PO₄.** 69 Gm. of NaH₂PO₄·1H₂O dissolved in distilled water and diluted to 250 ml.

PROCEDURE

Place 0.50 ml. of serum into a test tube cuvet and add 3.5 ml. of alkaline buffer. Mix gently and read in the photometer at or near 580 mμ using water as a reference. Add 0.10 ml. of acid reagent. Mix gently and read again. Proceed to the next sample. Determinations can be made on 0.1 ml. of serum using proportionally less of the reagents and reading in micro cuvets. In this laboratory 0.1 ml. serum, 0.7 ml. buffer and 0.02 ml. acid reagent was read in a 1 cm. micro cuvet at 580 mμ in a Beckman Model B Spectrophotometer.

Calculation is based on the 10.0 mg. per 100 ml. or 100 per cent retention standard put through the method as described.

\[
\text{mg. BSP per 100 ml. serum} = \frac{\text{mg. per 100 ml. of standard}}{\text{optical density of standard}} \times \text{optical density of serum.}
\]

\[
\text{Per cent retention (for 5 mg. per kilo dose)} = \frac{\text{Per cent retention standard} \times \text{optical density of serum}}{\text{optical density of standard}}
\]
The calculation of retention for the 2 mg. per kilo dose can be made by similar adjustments of standards. If the rate of disappearance of dye and per cent retention is desired, the formula of Nadeau (6) may be used since retention is proportional to concentration.

$$K = \frac{\log R_1 - \log R_2}{t_2 - t_1}$$

where $K$ is the velocity constant

$R_1$ is the per cent retention at time 1 ($t_1$) in minutes.

$R_2$ is the per cent retention at time 2 ($t_2$) in minutes.

**STUDY OF THE METHOD**

In order to determine the dissociation curve of BSP, 10 ml. of a 0.5 mg. per cent solution in 0.01M NaH$_2$PO$_4$ were placed in a 1 in. x 2 in. shell vial and then placed into a photometer so that absorbance at 580 m$\mu$ could be measured. Small increments of 1N NaOH were added to the reaction mixture from a Scholander microburet. Absorbance was measured with each change in pH. The data so obtained is plotted in Fig. 1. The $\nu$K for the dye is 8.8 and 98 per cent of color is obtained between pH 7.4 and 10.4. For purposes of analysis these pH limits were used in the determination of BSP in order to attain maximum color with minimum pH change. Table 1 shows the pH range achieved by this method as compared to the methods of Rosenthal (7), Gaebler (3), and Reinhold (4).

A series of optical density measurements was made on serums which contained no BSP, but which were clear, hemolyzed, jaundiced, or turbid in order to ascertain the effect of pH changes on optical

![Fig. 1. Dissociation curve of BSP.](image)
DETERMINATION OF SULFOBROMOPHTHALEIN IN SERUM

Table 1. pH Changes Obtained During BSP Measurements in Four Methods

<table>
<thead>
<tr>
<th>Method</th>
<th>No.</th>
<th>Mean</th>
<th>σ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reinhold</td>
<td>Serum*</td>
<td>35</td>
<td>8.31</td>
</tr>
<tr>
<td></td>
<td>Serum + NaOH</td>
<td></td>
<td>11.82</td>
</tr>
<tr>
<td></td>
<td>Δ pH</td>
<td></td>
<td>3.52</td>
</tr>
<tr>
<td>Gaebler</td>
<td>Serum*</td>
<td>36</td>
<td>8.31</td>
</tr>
<tr>
<td></td>
<td>Serum + NaOH</td>
<td></td>
<td>11.87</td>
</tr>
<tr>
<td></td>
<td>Δ pH</td>
<td></td>
<td>3.57</td>
</tr>
<tr>
<td>Rosenthal</td>
<td>Serum + NaOH</td>
<td>4</td>
<td>10.86</td>
</tr>
<tr>
<td></td>
<td>Serum + HCl</td>
<td></td>
<td>6.52</td>
</tr>
<tr>
<td></td>
<td>Δ pH</td>
<td></td>
<td>4.34</td>
</tr>
<tr>
<td>Proposed</td>
<td>Serum + alkaline buffer</td>
<td>36</td>
<td>10.48</td>
</tr>
<tr>
<td></td>
<td>Neutral mixture</td>
<td></td>
<td>7.12</td>
</tr>
<tr>
<td></td>
<td>Δ pH</td>
<td></td>
<td>3.36</td>
</tr>
</tbody>
</table>

*Data were obtained from 30 serums open to air 2-4 hours after clotting. These high values were not caused by hydrolysis of urea. pH’s were checked against buffer standards of pH 7.00 and 9.36 at 29°C.

density. The data shown in Table 2 indicate that greater optical density changes (calculated as per cent retention) occur in the Rosenthal (7), Gaebler (3), and Reinhold (4) methods where pH changes are uncontrolled (see Table 1). These changes introduce errors in the analysis which Gaebler and Reinhold (3, 4) correct by factors. The proposed method shows less interference when pH changes are controlled.

It was observed that the hue of the BSP at pH 10.3 was purple but became more pink when albumin was present. Figure 2 demonstrates

Table 2. Optical Density Changes Recorded as BSP in Clear, Hemolyzed, Jaundiced, and Turbid Serum Free of BSP in Order to Demonstrate the Effect of Interfering Factors

<table>
<thead>
<tr>
<th>Condition of serum</th>
<th>No.</th>
<th>Reinhold Mean</th>
<th>Reinhold Range</th>
<th>Gaebler Mean</th>
<th>Gaebler Range</th>
<th>Proposed Mean</th>
<th>Proposed Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clear</td>
<td>10</td>
<td>0.87</td>
<td>0-3.3</td>
<td>1.08</td>
<td>0-2.5</td>
<td>0.7</td>
<td>0-2.0</td>
</tr>
<tr>
<td>Hemolyzed*</td>
<td>8</td>
<td>2.6</td>
<td>1.3-6.7</td>
<td>3.4</td>
<td>1.6-7.0</td>
<td>1.5</td>
<td>0-3.4</td>
</tr>
<tr>
<td>Jaundiced*</td>
<td>8</td>
<td>1.1</td>
<td>(—) 1.6-3.8</td>
<td>1.4</td>
<td>0.3-5.5</td>
<td>0.4</td>
<td>0-2.5</td>
</tr>
<tr>
<td>Turbid*</td>
<td>2</td>
<td>3.9</td>
<td>(—) 3.6-4.1</td>
<td>2.9</td>
<td>1.6-4.2</td>
<td>0.7</td>
<td>0-2.1.2</td>
</tr>
<tr>
<td>Bilirubin Std.†</td>
<td>4</td>
<td>1.6</td>
<td>(—) 1.4-(—)1.8</td>
<td>1.3</td>
<td>1.1-1.7</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Hemoglobin Std.*</td>
<td>2</td>
<td>1.6</td>
<td>(—) 0.5-2.7</td>
<td>1.6</td>
<td>0.3-3.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

*27 to 138 mg. per 100 ml. hemoglobin by measurement. †1 to 30 mg. per 100 ml. bilirubin by measurement. ‡Grossly turbid. §2 and 5 mg. per 100 ml. bilirubin. ș13 and 137 per 100 ml. hemoglobin.
the spectral-absorbance curve of BSP at pH 10.3 without and with albumin. The maximum absorption occurs at 580 m\(\mu\) in the absence of serum and 594 m\(\mu\) in its presence. Furthermore, there was less absorbance in the latter case. When albumin solutions simulating serum samples were put through the analytical procedure, and where toluenesulfonate was omitted from the alkaline buffer, a loss of optical density was observed with increasing amounts of albumin (see Figs. 2 and 3).

In order to avoid this protein effect which could be troublesome since serum proteins vary widely in liver disease, a strong anion, p-toluenesulfonate, was added to the buffer. The albumin in serum is approximately 0.6 micromol per ml. or in the reaction mixture of this method 0.07 \(\mu\)mol. per ml. The BSP at 100 per cent retention is 0.12 \(\mu\)mol/ml. in serum or 0.015 \(\mu\)mol. per ml. in the reaction mixture. Albumin in normal serum is 5 times the molar concentration achieved by BSP at 100 per cent retention. p-Toluenesulfonate as added in the procedure reaches 29 \(\mu\)mol. per ml. in the final reaction mixture, which is 400 times the molar concentration of the albumin which would be

**Fig. 2.** Spectral-absorbance curve of BSP at pH 10.3 with and without albumin. Black dots = BSP; open squares = BSP + albumin + PTSA; open circles = BSP + albumin.
Fig. 3. Effect of albumin on optical density of BSP in serum. The abscissa represents Gm. per cent of an albumin solution containing BSP (25 per cent retention) which was run as a hypothetical serum (0.5 ml. albumin solution plus 3.5 ml. alkaline buffer).

present in the serum sample, and 900 times the BSP. When p-toluenesulfonate is present, the serum effect on the spectral absorbance curve almost disappears as shown in Fig. 2. The suppression of optical density also disappears. The sample of serum containing BSP measured with alkaline buffer free of p-toluenesulfonate gives 87 per cent of the optical density obtained with equal amounts of BSP in water or in serum as shown in Fig. 3. The standard curves obtained with serum BSP show the same difference (89 per cent) between those with and those without p-toluenesulfonate.

Comparison of the proposed method with the method of Reinhold (4) is shown in Table 3. The results are similar except for relative discrepancies in the lower concentrations and where marked hemolysis occurs.

DISCUSSION

The measurement of the simple dye BSP in the presence of albumin, bilirubin, hemoglobin, and turbidity can be difficult. Since measurements are most often made in serum, albumin is always present, more or less. In liver disease the albumin may vary from 0.5 to 5.0 Gm. per 100 ml. of serum. When turbidity is present, usually due to lipoproteins and lipids, some clearing may be achieved at high pH such as occurs when NaOH (3, 4, 7) is used for alkalinization. When acidification to low pH with HCl (7) occurs, the turbidity may recur and actually be more than that originally present in the serum sample.

1 Human serum albumin as provided by the American Red Cross for therapeutic use.
2 Data provided by Dr. John Reinhold of the Pepper Laboratory of the University of Pennsylvania.
3 It should be noted that hemoglobin, in serum which slowly becomes more alkaline, could change sufficiently so that hemoglobin corrections made for freshly drawn serum might not apply to serum which aged during transfer between laboratories.
This problem is accentuated by delaying readings between alkaline and acid reaction mixtures. Since BSP is measured at a wavelength (580 mμ) near where turbidity changes are measured, interference is not easily eliminated. The net effect of turbidity change from alkali to acid would be to give a falsely low BSP measurement. Hemoglobin present in serum resulting from hemolysis is converted in alkali to alkaline hematin. Over a broad pH range it causes changes depending upon time of exposure which can lead to errors. Bilirubin changes absorbance slightly between high and low pH so that jaundiced serums yield absorbance shifts with change of pH.

In the proposed method the pH shift is between 10.4 and 7.1. Over
this pH range there appears to be little or no absorbance change due to turbidity, jaundice, or hemolysis. Furthermore, in the presence of a large amount of p-toluenesulfonate no albumin effect is noted. When measurements are made as described the effects are essentially eliminated. Control of these factors favorably affect accuracy in the BSP measurement and reduce artifacts.

A series of serums were analyzed by the proposed method and by the method of Reinhold (4). The latter is Gaebler’s method with a third correction factor for hemoglobin. The proposed method obtained lower values in the low range where normal retentions occur. This may be due to less artifactual interference, although these readings are easily reproduced in this method. Furthermore, as shown above, serums with no BSP, even though hemolyzed, icteric, or turbid, do not give false readings.

Speed is achieved since no corrections for interfering substances need be made and no readings at two or three wavelengths need be made. Specificity appears to be increased by controlling pH.

The effects of albumin, as shown in Figs. 2 and 3, are negated by the anion p-toluenesulfonate when added in large amounts. It is assumed that albumin binds BSP and this binding is prevented by the presence of the p-toluenesulfonate.

**SUMMARY**

A method for the determination of BSP is presented which eliminates errors due to protein-binding with albumin and artifacts due to bilirubin, hemoglobin, and turbidity. Measurements at two or three wavelengths are thereby reduced to measurement at one wavelength. The method while simplified appears to be accurate, particularly in the low ranges.

**REFERENCES**


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*8 BSP mg. per 100 ml. = f (U565 - 1.28 U660) - 0.15 (U420 - 1.95 U660) where U420, U565, and U660 are absorbances at 420, 565 and 660 mμ, and f is the concentration of BSP absorbance at 565 mμ.*