Colorimetry of Hemoglobin in Plasma with 2,2'-Azino-di(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS)

Miyuki Takayanagi¹ and Tamotsu Yashiro²

Hemoglobin in plasma can be determined by the color-developing action of 2,2'-azino-di(3-ethylbenzthiazoline-6-sulfonic acid), which is oxidized to a colored form by a peroxidase-like effect of hemoglobin in the presence of hydrogen peroxide. Sensitivity, precision, and accuracy are discussed. The calibration curve is linear for hemoglobin concentrations up to 1 g/L; the minimum detectable concentration is 20 mg/L. The within-run precision (CV) was 2.39%, analytical recovery 101.8%. Interference from plasma proteins and lipids was eliminated by centrifuging the reaction mixture before measuring its absorbance at 410 nm.

Benzidine has long been used in determining the concentration of hemoglobin in plasma. In view of its carcinogenicity (1–3), safer reagents have been sought by many investigators, as exemplified by the tetramethylbenzidine method (4–6), the dicarboxidine method (7), and others (8). These methods have both merits and disadvantages from the standpoint of sensitivity, detection ranges, and ease of operation.

Deadman (9) and Bundgaard and Paby (10) have recently reported that 2,2'-azino-di(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) can be used to detect fecal occult blood with the same sensitivity as benzidine. We thus were prompted to use this chromogen in plasma. Hemoglobin shows a peroxidase-like effect in the presence of hydrogen peroxide, which oxidizes the ABTS reagent to a colored form. As others (11, 12) report, ABTS is readily oxidized to produce a green color.

Materials and Methods

**ABTS solution.** Dissolve 150 mg of ABTS (Wako Pure Chemical Industries, Osaka, Japan) in 10 mL of water and mix with 90 mL of acetic acid. Prepare this solution on the day of use.

**Diluted hydrogen peroxide solution.** Mix 1 mL of concentrated (300 g/L) hydrogen peroxide solution with 50 mL of water. This solution can be used for 6 to 8 h.

**EDTA solution.** Dissolve 200 mg of disodium EDTA in 100 mL of water.

**Phosphate buffer solution.** 0.1 mol/L, pH 7.6.

**Hemoglobin standard solution.** Wash 1 mL of packed erythrocytes twice with 2-mL portions of isotonic saline (NaCl, 9 g/L). Then hemolyse them by diluting to 10 mL with water and freezing at −30 °C. Thaw this hemolysate at room temperature, centrifuge (4000 × g, 10 min), and determine the hemoglobin in the supernate by the cyanmethemoglobin method. This solution is stable for at least three weeks at 4 °C. Dilute it to yield hemoglobin standards with concentrations ranging from 0.01 to 1.0 g/L, for use in preparing the standard curve.

**Effect of anticoagulants.** Five different specimens of hemoglobin-free plasma were each divided among three Venoject Tubes (Terumo Corp., Tokyo, Japan): VT-050H (containing sodium heparin as anticoagulant), VT-050NA (sodium EDTA), and VT-050CS (sodium citrate). Different volumes of the 1 g/L hemoglobin standard solution were added to each tube to yield final hemoglobin concentrations of 0.20, 0.50, and 0.80 g/L.

**Effect of bilirubin.** Bilirubin (Wako Pure Chemical Industries), 500 μg, was dissolved in 100 mL of 0.1 mol/L sodium carbonate and added to pooled plasma to obtain the desired final bilirubin concentrations.

**Hemoglobin assay procedure.** Pipet 2.5 mL of ABTS solution, 1 mL of EDTA solution, and 7 mL of phosphate buffer into test tubes, and mix well. Pipet 5 μL of each hemoglobin standard and sample into respective test tubes, and mix well. Add 1 mL of diluted hydrogen peroxide solution into each test tube and mix well. Incubate at room temperature for 90 min and centrifuge (1500 × g, 5 min). Within 20 min, measure the absorbances of the supernates at 410 nm.

Hünig et al. (11) report an absorbance maximum of oxidized ABTS at or near 420 nm. We found that, for our hemoglobin assay in plasma, the maximum absorbance of reaction mixtures was at 410 nm, so we used that wavelength.

For comparison, we also determined hemoglobin by the tetramethylbenzidine method as previously described (5).

**Results**

**Effect of pooled plasma.** Samples having the same concentration of hemoglobin showed equal absorbance when more pooled plasma was added to them (p < 0.05, by Student's t-test).

**Optimizing assay conditions.** We varied the ABTS concentration in the reagent between 0.5 and 1.5 g/L and hydrogen peroxide concentrations between 0.1 and 50 g/L. Samples showed greater absorbances with increasing ABTS concentration, and samples with a hydrogen peroxide concentration of 10 g/L showed the greatest absorbances. We optimized the assay system to include the 1.5 g/L ABTS solution and the 1.0 g/L diluted hydrogen peroxide solution. Varying the volume of added buffer between 5 and 10 mL changed the pH of the reaction mixture. Readings were more reliable between pH 2.5 and 2.8; although samples showed lower absorbance at this pH, they were more stable. We tested incubation temperatures between room temperature and 50 °C. At room temperature the ABTS oxidation reaction was suitably active, with minor extraneous reactions. At higher temperatures samples produced greater absorbances but were more unstable. We examined increasing the sample volume from 5 to 20 μL, and found that the system was optimized with 5 μL of sample.

**Precision.** Table 1 summarizes the results for precision. The data from which the CVs were calculated were obtained under routine operation by two research technologists.

**Time-course variation.** Figure 1 illustrates the time-

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Table 1. Precision of Hemoglobin Assay

<table>
<thead>
<tr>
<th>Hemoglobin</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>concn, mg/L</td>
<td></td>
</tr>
<tr>
<td>Within-run (n=40 each)</td>
<td></td>
</tr>
<tr>
<td>108</td>
<td>2.39</td>
</tr>
<tr>
<td>432</td>
<td>1.67</td>
</tr>
<tr>
<td>864</td>
<td>0.86</td>
</tr>
<tr>
<td>Between-run (n=40 each)</td>
<td></td>
</tr>
<tr>
<td>108</td>
<td>1.72</td>
</tr>
<tr>
<td>432</td>
<td>1.51</td>
</tr>
<tr>
<td>864</td>
<td>1.30</td>
</tr>
</tbody>
</table>

Samples were prepared by diluting plasma (citrate anticoagulant).

Fig. 1. Time-course of the ABTS/hemoglobin/H$_2$O$_2$ reaction
Hemoglobin concentrations in samples, 0.108 (□), 0.327 (▲), 0.763 (●), and 1.080 (●) g/L.

Fig. 2. Linearity of assay response to hemoglobin concentration

Table 2. Effect of Anticoagulants on Hemoglobin Recovery

<table>
<thead>
<tr>
<th>Anticoagulants</th>
<th>Added</th>
<th>Mean</th>
<th>SD</th>
<th>Mean recovery, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heparin</td>
<td>0.20</td>
<td>0.193</td>
<td>0.020</td>
<td>96.5</td>
</tr>
<tr>
<td>Citrate</td>
<td>0.20</td>
<td>0.200</td>
<td>0.019</td>
<td>100.0</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.20</td>
<td>0.195</td>
<td>0.021</td>
<td>97.5</td>
</tr>
<tr>
<td>Heparin</td>
<td>0.50</td>
<td>0.514</td>
<td>0.012</td>
<td>102.8</td>
</tr>
<tr>
<td>Citrate</td>
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<td>0.485</td>
<td>0.015</td>
<td>97.0</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.50</td>
<td>0.505</td>
<td>0.015</td>
<td>101.0</td>
</tr>
<tr>
<td>Heparin</td>
<td>0.80</td>
<td>0.815</td>
<td>0.015</td>
<td>101.8</td>
</tr>
<tr>
<td>Citrate</td>
<td>0.80</td>
<td>0.828</td>
<td>0.016</td>
<td>103.5</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.80</td>
<td>0.815</td>
<td>0.018</td>
<td>101.8</td>
</tr>
</tbody>
</table>

n=5 for each anticoagulant and each concentration.

Discussion

At the pH of the reaction mixture (about pH 2), plasma proteins and lipids gradually precipitate, and would interfere with the measurement of absorbance. However, the precipitate is easily removed by the centrifugation and therefore does not affect the determination.

As reported by Standefer and Vanderjagt (5), the absorbance of the reaction solution in the tetramethylbenzidine method increased for a while, then gradually decreased. In the present method the absorbance increased very gradually after incubation for 90 min (ΔA = 0.0005/min), but the measurement error was only 0.05 mg/L when measurement was made within 20 min after the incubation.

The normal reference interval for hemoglobin in plasma from healthy humans is usually indicated as being between 0 and 50 mg/L. Thus, for hemoglobin assay, the range up to 100 mg/L is clinically the most important. In the present method, concentrations of hemoglobin of about 100 mg/L produce absorbances of about 0.12 A, which can be measured very reproducibly. Very low concentrations of hemoglobin produced very low absorbances; we made such measurement in triplicate.

Compared with the tetramethylbenzidine method as de-
Hasegawa, hemoglobin over the quenching precise described the Samples Fig. We We considered the present method to be an improvement over other methods for determining the concentration of hemoglobin in plasma.

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References