An Evaluation of a Spectrophotometric Scanning Technique for Measurement of Plasma Hemoglobin

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ABSTRACT

The measurement of plasma hemoglobin is utilized in the evaluation of various hemolytic disorders. The purpose of this report is to illustrate the advantages of a direct spectrophotometric scanning technique for determination of plasma hemoglobin. This method does not require the use of carcinogenic reagents. The scanning method demonstrates linearity, precision, and sensitivity suitable for clinical use and is not affected by the presence of bilirubin.

Introduction

The measurement of free hemoglobin in plasma is frequently used in the evaluation of acute intravascular hemolytic disorders. Thus, the concentration of plasma free hemoglobin may be increased in conditions such as faulty intracardiac valvular prostheses, transfusion reactions, and paroxysmal nocturnal hemoglobinuria. The artifactual hemolysis incident to collection and processing of the blood specimen may elevate the actual hemoglobin level significantly and is one of the most important variables to control when evaluating the results of plasma hemoglobin determinations.

A number of methods have been employed for the determination of hemoglobin in body fluids. The spectrophotometric measurement of cyanomethemoglobin following conversion of most forms of hemoglobin to cyanomethemoglobin using Drabkin’s reagent has been recommended as the standard method for whole blood.10 The measurement of plasma hemoglobin requires quite sensitive methods since the concentration of hemoglobin in plasma is normally less than 0.05 percent of the whole blood concentration. An early method for hemoglobin measurement used the peroxidase activity of hemoglo-
bin to catalyze the peroxide oxidation of benzidine to a colored product.\textsuperscript{15} There have been several modifications of the benzidine method with variations in the assay conditions\textsuperscript{2,7} and in the substrates employed.\textsuperscript{12,13} The use of analogues of benzidine, such as o-tolidine and 3,3', 5,5'-tetramethylbenzidine, was proposed owing to the restrictions on benzidine because of its carcinogenicity.\textsuperscript{6} Plasma hemoglobin has also been quantitated using nephelometry.\textsuperscript{5}

Direct spectrophotometric scanning of plasma samples has also been used to determine the level of hemoglobin and hemoglobin pigments.\textsuperscript{1,3,8,9,11} The spectrophotometric method of Harboe is a commonly used method for measuring plasma hemoglobin, but it is strongly subject to interference by elevated levels of bilirubin.\textsuperscript{8} The method of Blakney and Dinwoodie is not subject to various chemical interferences, such as elevated bilirubin levels, nor does it use carcinogenic reagents.\textsuperscript{1} This report is an evaluation of the Blakney and Dinwoodie spectrophotometric scanning method as a practical and reliable technique for the measurement of plasma free hemoglobin in the clinical laboratory.

Methods

PRINCIPLE

The fractional absorption of a portion of the 578 nm band of oxyhemoglobin is used in conjunction with the absorption coefficient of oxyhemoglobin to relate absorption to hemoglobin concentration. The use of the fractional absorption of oxyhemoglobin allows for correction of background absorbance owing to substances whose absorbance is linear (but not necessarily constant) in the limited wavelength range of 562 to 598 nm. The fractional absorbance of oxyhemoglobin at 578 nm is thus proportional to the concentration of hemoglobin even in the presence of interferences from bilirubin.

SAMPLE COLLECTION

An 18 gauge needle with attached tubing from an infusion set is used for sample collection. A tourniquet is placed lightly around the upper arm and the antecubital vein is carefully punctured to minimize trauma. The tourniquet is released and the tubing is clamped with a hemostat as soon as the blood return is observed. The stoppers of a three ml red vacutainer tube containing no anticoagulant and a five ml green heparinized vacutainer tube are removed. The clamp is released, and blood is allowed to drip into the three ml vacutainer first. Indirect collection minimizes vigorous expansion of the blood into evacuated tubes which may contribute to \textit{in vitro} hemolysis.\textsuperscript{14} The free end of the tubing is then transferred into the heparinized tube and five ml of the freely flowing blood is collected. The tubing is clamped off and the needle withdrawn. The three ml vacutainer serves to clear the local vascular area of hemoglobin released artifactualy owing to the venipuncture, and this tube may be discarded or the serum used for other analyses. The heparinized tube is recapped immediately and tipped gently three to five times. The heparinized blood is centrifuged for 10 min at 1000 \times g. The supernatant plasma is drawn off with a Pasteur pipet without disturbing the buffy layer and delivered into a second tube. The plasma is then recentrifuged for 20 min at 1600 \times g. The supernatant plasma is transferred into a third tube. Stability of the plasma sample for hemoglobin determination is 24 hrs at 4° or up to three weeks when stored at \textminus20°C.

EQUIPMENT

Zeiss PMQII Spectrophotometer (band pass width equal to 0.9 to 1.0 nm) or equivalent spectrophotometer.

PROCEDURE

Approximately 1.0 ml of untreated plasma is transferred by Pasteur pipet to a
quartz microcuvet* and deionized water is similarly transferred to a second quartz microcuvet. The wavelength on the spectrophotometer is set at 562 nm and slit width at 0.015 mm. The absorbance is set to zero with the water blank. The absorbance of the sample is then obtained. The wavelength is then reset at 578 nm and the absorbance set to zero with the water blank. The absorbance of the sample is read and recorded. The wavelength is then reset at 598 nm and the absorbance set to zero with the water blank. The absorbance of the sample is obtained.

**Calculation**

The fractional absorption of oxyhemoglobin at 578 nm is calculated from the total absorption at 578 nm by subtracting the tangential baseline absorption at 578 nm determined by drawing a linear tangent between 562 nm and 598 nm (figure 1). A calibration factor of 155 (mg per dl) was calculated from the millimolar absorption coefficient of oxyhemoglobin at 578 nm of 61.48, a molecular weight of 64,456, and a fractional absorption of oxyhemoglobin standard at 578 nm of 67.4 percent

\[
\left( \frac{\Delta A}{A} \times 100 \right)
\]

\[
\frac{A_{562} - A_{598}}{2.25} = X
\]

\[
A_{562} - X = Y
\]

\[
(A_{578} - Y) \times 155 = \text{plasma hemoglobin in mg per dl}
\]

\[
Y = \text{calculated tangential baseline at 578 nm}
\]

\[
(A_{578} - Y) = \text{fractional absorption of oxyhemoglobin}
\]

For comparison, plasma hemoglobin was also determined by the methods of Crosby and Furth, Harboe, and by use of Drabkin's reagent.

**Results and Discussion**

The spectrophotometric scanning method for plasma hemoglobin is simple and rapid. Satisfactory results have been obtained with the Zeiss PMQII Spectrophotometer which has a band pass width of 1.0 nm. This method does not yield acceptable results when a spectrophotometer of band pass width 8.0 nm or greater is used. Prior treatment of the patient sample is not required since any reduced hemoglobin is converted to oxyhemoglobin on exposure to room air.

A comparison of the spectrophotometric scanning method and of the modified Drabkin’s method is shown in figure 2 (figures 2A and 2B, respectively). The modified Drabkin’s method is linear over the range of 0 to 325 mg per dl. This range approximates the semi-quantitative description of 0 to 4+ hemolysis. The spectrophotometric scanning method is linear from 5 to 150 mg per dl. This method had previously been reported to obey Beer’s Law over the range of 2.5 to

*Type 9Q, 10 mm, Precision Cells, Inc., Hicksville, NY.*
FIGURE 2. Linearity of the spectrophotometric scanning method (2A) and the modified Drabkin's method (2B) for determination of plasma hemoglobin.

100 mg per dl. Although the scanning method is not accurate or precise at very low concentrations of hemoglobin (less than 2.0 mg per dl), it is sufficiently sensitive and linear for routine clinical purposes.

The scan method was compared to the benzidine method in a 46 patient split sample correlation study. A linear least-square regression analysis showed a correlation coefficient of 0.97. The two methods demonstrated comparable results over the range of 0 to 123 mg per dl plasma hemoglobin (figure 3). Statistical analysis of the correlation data by the Student's t-test for paired data indicated that the correlation between the two sets of values was highly significant (0.3 > p > 0.2) and that there was no significant difference between individual means.

The precision of the spectrophotometric scanning method is shown in table I. Interrun precision yielded coefficients of variation of 5.9 percent at the 3.6 mg per dl level and 4.3 percent at the 50.6 mg per dl level. Intrarun precision showed coefficients of variation of 5.0 percent at the 3.6 mg per dl level and 3.0 percent at the 50.9 mg per dl level.

The spectrophotometric method of Harboe is a commonly used laboratory method for plasma hemoglobin. In this method, hemoglobin concentration is cal-

TABLE I
Interrun and Intrarun Precision Data for Plasma Hemoglobin Determined by Spectrophotometric Scan

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>MEAN mg per dl</th>
<th>SD mg per dl</th>
<th>CV Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interrun Normal</td>
<td>20</td>
<td>3.58</td>
<td>0.21</td>
<td>5.87</td>
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<tr>
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<td>20</td>
<td>50.61</td>
<td>2.16</td>
<td>4.27</td>
</tr>
<tr>
<td>Intrarun Normal</td>
<td>20</td>
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<td>0.18</td>
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<tr>
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<td>20</td>
<td>50.87</td>
<td>1.54</td>
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</table>

FIGURE 3. Correlation between the spectrophotometric scanning method (y) and benzidine method (x) for the determination of plasma hemoglobin. $\bar{y} = 1.15x - 2.91$, $r = 0.97$, $x = 24.77$ mg per dl, $\bar{y} = 25.90$ mg per dl, n = 46.
HEMOGLOBIN (MG/OL)

HARBOE METHOD

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HEMOGLOBIN (MG/OL)

BENZIDINE METHOD

Figure 4. Correlation between the Harboe method (y) and benzidine method (x) for the determination of plasma hemoglobin.

culated from the oxyhemoglobin absorption at the Soret band (415 nm) and corrected for background absorption at 380 nm and 450 nm. This background correction cannot be applied to samples with elevated bilirubin concentrations since the absorption of bilirubin is non-linear between 380 and 450 nm. Correlation of the Harboe method to the benzidine method is shown in figure 4. A linear least-squares regression analysis yielded a correlation on coefficient of 0.95; this may be due, in part, to the interference of elevated levels of bilirubin.

The effects of varying concentrations of bilirubin on the measured plasma hemoglobin level using the Harboe method, the benzidine method, and the direct spectrophotometric scanning method are shown in table II. In general, bilirubin produced the greatest interference when hemoglobin was determined by the Harboe method. Results from the scan method demonstrated better agreement with the benzidine method results. Of the three methods, the scanning method is least affected by bilirubin concentration because the background absorption for bilirubin is linear, although not constant, between 562 nm and 598 nm. Similarly, the method should not be affected by the turbidity of the sample.

Conclusions

The spectrophotometric scanning method of Blakney and Dinwoodie is a simple and rapid technique for determination of plasma hemoglobin. The method is linear from 5 to 150 mg per dl plasma hemoglobin and is sufficiently sensitive for routine clinical use. Split-sample correlation with plasma hemoglobin results obtained using benzidine demonstrated an excellent correlation between the two methods ($r = 0.97, N = 46$). The scan method demonstrates acceptable precision and is not significantly interfered with by elevated bilirubin levels. The scan method requires a narrow band pass width spectrophotometer but does not use hazardous chemicals. Therefore, the spectrophotometric scanning method is a practical and economical technique for determination of plasma hemoglobin.

TABLE II

The Effect of Bilirubin Concentration on Three Methods for Determination of Plasma Hemoglobin

<table>
<thead>
<tr>
<th>Total Bilirubin (mg/dl)</th>
<th>Plasma Hemoglobin (mg/dl)</th>
<th>Benzidine</th>
<th>Scan</th>
<th>Harboe</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.8</td>
<td>2.5</td>
<td>0.8</td>
<td>1.5</td>
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<tr>
<td>0.8</td>
<td>7.2</td>
<td>6.2</td>
<td>5.8</td>
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</tr>
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<td>0.8</td>
<td>2.9</td>
<td>3.1</td>
<td>3.9</td>
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<td>2.8</td>
<td>57.0</td>
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References


