Measuring Hemoglobin in Plasma by Reaction with Tetramethylbenzidine

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The reaction of hemoglobin with tetramethylbenzidine was studied with a centrifugal analyzer. We found that the hemochromogen reaction was significantly influenced by albumin. Increasing amounts of albumin apparently stimulate the hemochromogen reaction during the earlier part of an incubation and inhibit it later in the incubation. However, during an intermediate period, when the crossover from stimulation to inhibition occurs, the assay is independent of albumin and bilirubin concentrations. Increasing the temperature of incubation or the hydrogen peroxide concentration, or decreasing the tetramethylbenzidine concentration, shortens the crossover time. By adjusting these variables the method can be used with most instrumentation. We developed procedures for use on a centrifugal analyzer or for manual assay (reaction times, 4 and 20 min, respectively). Accuracy, as indicated by a comparison with a direct hemoglobin method ($r = 0.990$) and by recovery experiments, was excellent. The CV for the automated assay was less than 3%. Plasma collected in citrate and heparin showed good recovery of hemoglobin; recovery in EDTA was poorer.

Additional Keyphrases: centrifugal analyzer • variation, source of • albumin • reference interval

Measurement of plasma hemoglobin is useful in assessing the degree of acute intravascular destruction of erythrocytes for further medical treatment and evaluation in conditions where hemolysis follows acute mechanical, chemical, immunological, or radiological (1) disruption of cells. Benzidine has most commonly been used for plasma hemoglobin measurement, but its use has been curtailed because of its carcinogenicity.

Direct spectrophotometric assays may be used to assay hemoglobin, but these require large samples and are substantially influenced by high concentrations of bilirubin, which shows considerable overlap with the absorbance curve of hemoglobin (2). Recently, in two reports, tetramethylbenzidine (TMB) was substituted for benzidine for measuring hemoglobin by manual methods (3, 4). Because of the presence of certain inhibitors of the color development in sera, one method (3) requires that each patient and control be measured in triplicate to obtain each result. The other method, although less labor intensive, gives results linear with concentration up to only 300 mg/L (4); moreover, its accuracy was assessed only on samples of hemoglobin in a plasma "surrogate" rather than a true plasma matrix.

To develop a reliable, less labor-intensive method having a wide range of linearity, we tried to improve the assay by using a centrifugal analyzer and various concentrations of sample, reactants, and sample medium, and by varying temperature and time of incubation.

Contrary to previous thought (3), we found that albumin was the main serum interferent with color development, and that this effect was largely dependent upon time of incubation.

These findings enabled us to develop for the centrifugal analyzer a rapid method that involves a small sample and is insensitive to high concentrations of bilirubin; we also developed a back-up manual method.

Materials and Methods

$3,3',5,5'$-Tetramethylbenzidine (mol. mass 240.35, AR grade; Aldrich Chemical Co., Milwaukee, WI 53233). For the centrifugal analyzer assay, dissolve 5 g in 100 mL of glacial acetic acid/water (90/10, by vol) to make a stock solution (stable for three months when stored in a brown bottle at 4 °C). Dilute one volume of the stock solution 40-fold with the acetic acid/water solution to make the routine TMB working reagent (1.25 g/L); this solution is stable for 4 h at room temperature. Other concentrations of working color reagent were also prepared for comparison.

For the manual assay, dissolve 2.5 g of TMB in 500 mL of the acetic acid/water solution; this working solution is stable for three months if stored in a brown bottle at 4 °C.

Hydrogen peroxide, 30 g/L. For the centrifugal analyzer assay, dilute this solution 40-fold with water to make the working solution (0.75 g/L); the working solution is stable at room temperature for 4 h.

For the manual method, dilute the 30 g/L solution 30-fold with water to make a working solution of 1 g/L.

Dilute acetic acid for manual assay. Add 100 mL of glacial acetic acid to 500 mL of water; diluent is stable at room temperature indefinitely. If the concentration of acetic acid in the reaction mixture decreases to <100 mL/L, the solution becomes unstable and turns blue.

Hemoglobin calibrators. Erythrocytes were lyzed by diluting with water. The resulting soluble stock hemoglobin solution (approx. 10 g/L) was analyzed by a cyanmethemoglobin technique (3). This solution was diluted with either hemoglobin-free serum or saline to make calibrators in concentrations ranging between 0.02 and 2.5 g/L. To ensure accuracy, we assayed aliquots of these standards for hemoglobin by a direct method (5). Calibrators were stable for up to three months when kept refrigerated in brown bottles.

Human albumin, 250 g/L (Michigan Department of Public Health, Lansing, MI 48909; a similar solution can be obtained from Travenol Laboratories, Glendale, CA 91202). This solution was diluted with saline or hemoglobin-free serum to make albumin solutions of desired concentrations. Endogenous albumin in serum was determined by a bromocresol green method (SMA; Technicon Corp., Tarrytown, NY 10591).

Anticoagulants. Different hemoglobin-free plasmas were selected without conscious bias, six collected in blue-top (sodium citrate), six in green-top (sodium heparin), and six in lavender-top (potassium EDTA) Vacutainer Tubes (Becton Dickinson and Co., Rutherford, NJ 07070). Each of the samples was divided into three aliquots, and different amounts of hemoglobin calibrator (10 g/L) were added to each aliquot to give final hemoglobin concentrations of 0.29, 0.57, and 1.15 g/L.

Patient's samples. Serum was collected with no anticoagulant, or plasma was collected in green-top Vacutainer Tubes, from patients undergoing open heart surgery. The exact amount of heparin in the samples is unknown because these patients are treated with heparin during surgery. All
samples that were without jaundice were assayed by a direct hemoglobin method (5) for correlation studies.

**Bilirubin** (Sigma Chemical Co., St. Louis, MO 63178), 500 mg dissolved in 100 mL of 0.1 mol/L sodium carbonate. Aliquots were added to serum to obtain the desired final bilirubin concentrations.

**Lipemic samples.** Chylomicrons were concentrated by ultracentrifugation (Airfuge; Beckman Instruments, Brea, CA 92621). Aliquots were added to serum to give triglyceride concentrations of 1.5, 3.25, and 7.5 g/L for turbidity studies.

**Hemoglobin assay by centrifugal analyzer.** We used a Centrifichem 500 (Baker Instruments, Pleasantville, NY 10570) for both pipetting and analysis. For routine analysis the instrument was set as follows:

- Zero (T0), 003 s
- ΔT, 04:00 min
- Abnormal absorbance, 3.0 U
- Blank, auto
- Test mode, term
- Print out, conc
- Conc. factor, 0100
- No. of prints, 01
- Temp., 37 °C
- Filter, 620
- Test code, 00

The Centrifichem 500 system can pipet two reagents and samples plus diluent into a 30-place transfer disc. Into the sample well are pipetted 100 µL of working TMB, 5.0 µL of sample, and 45 µL of diluent (water). Into the separate reagent well, 250 µL of working hydrogen peroxide solution is pipetted. A water blank is included in position No. 0, and a reagent blank in position No. 1. We placed duplicate 1.0 g/L calibrators in positions 2 and 3. The centrifugal analyzer automatically mixes and transfers reagents from the 30-place disc to 30 corresponding cuvets, where absorbance readings are taken at 3 s (T0) and 4 min (ΔT) after mixing. The absorbances are read against the water blank and corrected for drift in the reagent blank before calculations, based on the calibrator concentration, are completed.

We also investigated different temperatures, and times for reading absorbance, as indicated in the results.

**Manual assay.** Pipet 1 mL of TMB solution (5.0 g/L) into each of several 16 × 150 mm test tubes. Pipet 20 µL of 1 g/L calibrator (duplicates) and control sera into appropriately labeled 16 × 150 mm test tubes. Include one test tube with TMB solution but without sample, as a reagent blank. At 10-s intervals, add 1 mL of working hydrogen peroxide solution (1.0 g/L) to each test tube, and mix well. Incubate the solutions for 20 min at room temperature. Pipet 10 µL of diluent into each test tube in 10-s intervals, in the same sequence that the hydrogen peroxide was added. Mix each by inversion. With a digital-type spectrophotometer that can read up to 2.999 absorbance units, read without delay the absorbance at 660 nm of each sample in the same order that the diluent was added. Subtract the blank reading from each standard, sample, and control to obtain the absorbance difference. Average the absorbance differences of the duplicate calibrators and calculate the concentration of hemoglobin in each unknown.

**Wavelength.** Although the absorbance peak for this reaction is near 660 nm, 620 nm is also suitable for the assay (4). We used 620 nm for the Centrifichem because the instrument does not contain a 660-nm filter and the higher sensitivity was unnecessary; 660 nm was used for the manual method because that gives maximum sensitivity.

**Hemoglobin assay by a direct method** was performed as previously described (5).

### Results

**Effect of albumin on absorbance.** The change in absorbance with time is dependent on the concentration of both hemoglobin and albumin. With a constant amount of hemoglobin, samples containing more albumin showed more absorbance at 2 min and less absorbance after 6 min than did samples containing less albumin (Figure 1). A crossover in absorbance occurs at 4 min, when the change in absorbance can be considered independent of albumin concentrations. Hence, the timing of the reaction sequence is critical. Use of a centrifugal analyzer facilitates good timing control. This effect of albumin was observed whether albumin was added to samples containing low concentrations of endogenous albumin, or albumin was dissolved in saline containing hemoglobin, or only endogenous albumin was present.

**Effect of temperature and concentration of reactants.** We investigated final TMB concentrations in the reaction mixture between 0.31 and 2.5 g/L and hydrogen peroxide concentrations between 0.25 and 1.0 g/L. As the TMB concentration was increased, the crossover between apparent stimulation and inhibition occurred later in the incubation. Increasing the incubation temperature between 25 and 37 °C or increasing the hydrogen peroxide concentration caused the crossover to occur earlier. Increasing the sample volume ratio in the final reaction mixture from 1/200 to 1/80 caused the crossover to occur later, because of the increase in albumin concentration (150 g/L) in the larger sample (1/80) vs 60 g/L in the smaller sample (1/200) that the system was optimized to assay.
Linearity and sensitivity. Figure 2 indicates that the automated method responds linearly to hemoglobin concentrations between 0.02 and nearly 2.5 g/L. The range of linearity in the manual method was similar. Figure 2 also indicates no overlap between hemoglobin concentrations of 0 and 0.02 g/L for each of duplicate samples. Thus, the detection limit of this method is 0.02 g/L.

Reagent blank. As Figure 3 shows, when the final concentration of TMB in the reaction mixture exceeds 2.5 g/L, there is a substantial change in absorbance, even when no sample is added. This effect does not interfere with good linearity after the blank is subtracted from each sample, but it does cause poorer reproducibility in the low hemoglobin range. Thus, for automated analysis it is preferable to use low concentrations of TMB.

Reproducibility. Between-run coefficients of variation (CV) with the CentrifiChem were 2.4, 2.7, and 2.7% for serum concentrations of hemoglobin of 0.27, 0.55, and 1.07 g/L, respectively, for 12 consecutive assays. Within-run CVs for 10 replicates of each of these samples were 2.2, 1.9, and 2.0%, respectively.

Between-run variability for the manual method was 2.8, 2.2, and 1.8% for sera with hemoglobin contents of 0.30, 0.55, and 1.05 g/L, respectively, for 10 assays performed by the same technician. When the assay was performed by several technicians in the routine laboratory rotation, between-run reproducibility dropped substantially, with a CV of 7% for a hemoglobin of 0.9 g/L, and 9% for 0.25 g/L.

Effect of bilirubin and turbidity. When bilirubin to give final concentrations of 7, 15, 30, 60, 125, and 250 mg/L was added to sera containing hemoglobin concentrations of 0.27, 0.64, and 1.3 g/L, analytical recoveries with the CentrifiChem varied between 95 and 108%, with random fluctuation.

Turbidity caused by chylomicrons (triglycerides at 1.50, 3.25, and 7.5 g/L) resulted in hemoglobin recoveries of 100, 95, and 94%, respectively, for sera containing hemoglobin concentrations of 0.5 and 1.0 g/L and assayed with the CentrifiChem. The serum with the greatest concentration of triglycerides was extremely turbid, yet its effect on the hemoglobin result would not be clinically important.

Anticoagulants. Table 1 indicates that hemoglobin added to plasma containing heparin showed the best average recovery. Plasma containing citrate showed a slightly greater recovery, but the difference from the values with heparin was not clinically significant. Recovery was less and reproducibility was poorer in EDTA-treated samples.

Table 1. Effect of Anticoagulants on Hemoglobin Recovery

<table>
<thead>
<tr>
<th>Hemoglobin, g/L</th>
<th>Added</th>
<th>Mean recovered (and SD)</th>
<th>Mean recovery, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heparin</td>
<td>0.29</td>
<td>0.285 (0.012)</td>
<td>99</td>
</tr>
<tr>
<td>Citrate</td>
<td>0.29</td>
<td>0.30 (0.019)</td>
<td>103</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.29</td>
<td>0.26 (0.021)</td>
<td>90</td>
</tr>
<tr>
<td>Heparin</td>
<td>0.57</td>
<td>0.56 (0.020)</td>
<td>98</td>
</tr>
<tr>
<td>Citrate</td>
<td>0.57</td>
<td>0.60 (0.021)</td>
<td>105</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.57</td>
<td>0.53 (0.039)</td>
<td>93</td>
</tr>
<tr>
<td>Heparin</td>
<td>1.15</td>
<td>1.14 (0.036)</td>
<td>99</td>
</tr>
<tr>
<td>Citrate</td>
<td>1.15</td>
<td>1.16 (0.034)</td>
<td>101</td>
</tr>
<tr>
<td>EDTA</td>
<td>1.15</td>
<td>1.06 (0.075)</td>
<td>93</td>
</tr>
</tbody>
</table>

n = 6.

Fig. 2. Linearity of assay response to hemoglobin concentration

(n = 14, r = 0.9997, slope = 0.73, intercept = 0.004). The line was calculated from points between 0.02 and 1.25 g/L. Bars indicate the range of duplicates. The assay was performed at 37 °C, timed for 4 min, with concentrations of sample and reactants as described for Fig. 1.

Fig. 3. Blank reaction vs absorbance

(Θ); (O), (X), (Δ), represent final concentrations of TMB in the reaction mixture of 2.5, 1.25, 0.62, and 0.31 g/L, respectively. Other conditions as in Figs. 1 and 2.
correspondence with the direct method for hemoglobin determination (Figure 4) and by the recovery in plasma samples treated with either citrate or heparin (Table 1).

The reference interval for plasma hemoglobin is usually given as 0–0.025 g/L, although values <0.1 g/L are rarely clinically important. For most clinical indications, samples can be run as singles, not duplicates, because concentrations of hemoglobin near 0.1 g/L produce absorbances near 0.08 A, which can be measured with a great degree of reproducibility by instruments such as the CentrifIChem. But as the absorbance decreases to very low values, the reproducibility of the instrument will decrease accordingly, causing poorer differentiation between samples with small concentration differences. Therefore, we recommend assaying samples and blanks in duplicate when hemoglobin concentrations between 0 and 0.1 g/L are of importance.

Although previous investigators used TMB at 5.0 g/L in the final mixture (4), our data indicate 0.31 g of TMB per liter allows good sensitivity in the CentrifIChem procedure, while maintaining a low reagent blank.

In the manual procedure, the reaction does not reach an endpoint. It is therefore necessary to dilute the reaction mixture five- to 10-fold to reduce the rate of reaction and to avoid constant upward drift in the spectrophotometer. This requires that initial concentration of TMB be about 2.5 g/L so that enough absorbance will be developed to determine accurately the hemoglobin concentrations after the diluent is added.

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References