Determining Methemoglobin in Blood by Zero-Crossing-Point First-Derivative Spectrophotometry

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We determined methemoglobin in blood by zero-crossing-point first-derivative spectrophotometry. After lysis of erythrocytes, hemoglobin was converted into oxyhemoglobin and the first-derivative spectrum was recorded between 405 and 425 nm. At the exact point where the first-derivative spectrum of oxyhemoglobin was zero ("zero-crossing point"), the first-derivative value of oxyhemoglobin and methemoglobin mixture was proportional to the methemoglobin concentration. The standard curve was linear for all proportions of methemoglobin. Within-assay precision (CV) was 3.4% for a 20% methemoglobin content. Correlation with results by the Evelyn and Malloy method was very good for high proportions of methemoglobin (>10%), but the proposed technique was far better for low methemoglobin percentages because of its linearity, its high sensitivity, and its low detection limit.

Additional Keyphrases: erythrocytes • hemoglobin • Evelyn-Malloy method compared

The methemoglobin content in blood is difficult to determine for three reasons. First, the spectral properties of the different hemoglobin species are very similar: the Soret band maximum is found at 406, 414, and 430 nm for methemoglobin, oxyhemoglobin, and hemoglobin, respectively, and their spectra overlap considerably in the 500- to 600-nm region (1). This problem can be solved by using multiwavelength spectrophotometry (2) or by direct measurement of absorbance at 630 nm, by using a small band characteristic of methemoglobin (1).

But other substances can interfere with these methods; for instance, methylene blue, which is widely used in emergency treatment of methemoglobinemia, also absorbs strongly in the 630-nm region and can be mis-measured as methemoglobin (3). Similarly, light-scattering effects in samples from patients receiving parenteral treatment with lipid emulsion in intensive-care departments can result in abnormally high results (4). Consequently, the concentration of methemoglobin in blood is generally determined in the 630-nm region by a differential method, or by derivative spectrophotometry. In the differential method of Evelyn and Malloy (5)—or adaptations of it (6, 7)—the characteristic band near 630 nm completely disappears when the addition of potassium cyanide converts methemoglobin into cyanmethemoglobin, which does not absorb at this wavelength. The resulting decrease in absorbance is proportional to the original concentration of methemoglobin. This concentration can also be determined by derivative spectrophotometry (8), because it is a powerful tool for analysis of overlapping spectra and for quantification of substances in turbid samples (9, 10). Theoretically, these techniques are very specific, avoiding interference from other hemoglobinins as well as from colored substances or turbidity. Unfortunately, they lack accuracy at low concentrations, because the absorptivity of methemoglobin near 630 nm is weak.

Numerous compounds, including ozone, potassium chloride, nitrites, antipyrine, and chloroquine, can oxidize hemoglobin and produce methemoglobin. People who are regularly in contact with such compounds will be susceptible to induction of methemoglobin. Because of the need for a simple, reliable, and sensitive method for determining the concentration of this analyte, we used the Soret band for methemoglobin in our investigation. The absorptivity of this molecule near 400 nm is 50-fold greater than in the 630-nm region (1). Because of the strong overlap of the Soret bands of different hemoglobin species, we used derivative spectrophotometry. Owing to the difficulties encountered during preliminary work, we finally selected zero-crossing-point first-derivative spectrophotometry (9, 11): at the wavelength of the absorption maximum of a compound, the value of its first derivative is zero, whatever the compound's concentration. For a mixture of two compounds, the first-derivative value at this wavelength will depend only on the concentration of the second component.

Consequently, after the conversion of hemoglobin into oxyhemoglobin, the zero-crossing-point first-derivative method allows the quantification of methemoglobin at the wavelength where the first-derivative spectrum of oxyhemoglobin is zero.

Materials and Methods

Materials

All reagents were analytical grade, from Prolabo, Paris, France. For spectrophotometric measurements, we used a Gilford 2600 spectrophotometer (Ciba-Corning, 78110 le Vesinet, France) with a Hewlett-Packard 7225 B recorder. To prepare a 160 g/L methemoglobin standard, we mixed 10 mL of potassium ferricyanide (200 g/L in distilled water) with 10 mL of pooled blood from healthy subjects, incubated the mixture at room temperature for 10 min, then centrifuged the sample and removed the supernatant liquid. We washed the remaining cells twice with isotonic saline solution (9 g/L NaCl) and lysed the erythrocytes by adding cold distilled water (about 0.9 volume) and freezing and thawing twice. After centrifugation, we determined the hemoglobin concentration with Drabkin's reagent and adjusted the concentration to 160 g/L. Finally, we diluted 250 μL of hemolysate exactly to 100 mL with phosphate buffer, pH 6.6 (12).

To prepare a 160 g/L oxyhemoglobin standard, we centrifuged blood cells from a healthy subject, washed them twice with isotonic saline solution, and lysed the erythrocytes as described above. After centrifugation, we determined the exact hemoglobin concentration with Drabkin's reagent and adjusted it to 160 g/L. We then mixed 250 μL of hemolysate and 500 μL of hydrogen peroxide (30 g/L) and without delay diluted this to 100 mL with phosphate buffer, pH 6.6.

We prepared calibration solutions by mixing various volumes of the methemoglobin and oxyhemoglobin stan-

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dards to obtain methemoglobin proportions in the range of 0% to 100% (0 to 160 g/L).

Procedures

**Determining the proportion of methemoglobin.** At the same time, prepare a solution A1 with a control blood from a healthy subject and solutions B1 and B2 with patient's blood. For each solution, dilute 25 µL of blood with 2 mL of cold distilled water. Shake and wait for 10 min to lyse the erythrocytes. Then add 25 µL of hydrogen peroxide (30 g/L) to solutions A1 and B1, and 10 µL of sodium nitrite solution (50 g/L in distilled water) to solution B2. Mix and dilute to exactly 10 mL with phosphate buffer, pH 6.6. Then prepare solution A2, diluting 5 mL of solution A1 with 5 mL of phosphate buffer, pH 6.6. Record the spectra for solutions A1, B1, and B2 between 405 and 425 nm, using solution A2 as a blank in the reference channel. To record the first-derivative spectra, smooth the curves by using a 15-point "derivative window." This window gives the best results for optimizing the signal-to-noise ratio.

Adjust the recorder scale according to the solution concentration: 0.25 A/nm full-scale for methemoglobin proportions >10% and 0.025 A/nm full-scale for lesser concentrations. Measure on the graph the derivative value Δ1 for solution B1 and Δ2 for solution B2 at the exact wavelength where the derivative curve for solution A1 crosses zero. Calculate the percentage methemoglobin according to the formula:

\[
\text{methemoglobin} \% = (\Delta_1/\Delta_2) \times 100
\]

**Determining the concentration of methemoglobin.** To determine the concentration of methemoglobin, compare Δ1 with the derivative values obtained under the same conditions as for the calibration solutions (for this approach, it is not necessary to prepare solution B2).

**Results**

**Linearity.** To test the linearity of this method, we constructed standard curves relating the concentration C (g/L of methemoglobin) to the first-derivative value Δ1 (absorbance/nm) for 10 calibration solutions with methemoglobin contents within the range of 16 to 160 g/L (10% to 100% methemoglobin) and for 11 solution preparations with 0% to 10% methemoglobin (0 to 16 g/L). The linear regression equations are respectively:

\[
C = 1174 \Delta_1 + 1.2 (r = 0.999)
\]

\[
C = 1180 \Delta_1 - 57 (r = 0.999)
\]

**Sensitivity.** A difference of 1 g of methemoglobin per liter produced a change in the first-derivative value of 8 × 10⁻⁴ A/nm. Plotting the curves on an expanded scale, we determined the imprecision in graphical determination of derivative values to be about 1.5 × 10⁻⁴ A/nm. From this imprecision and the measured intercept of the curves, we determined the detection limit as 0.5 g of methemoglobin per liter. This value can be lowered by using larger blood samples.

**Precision.** Within-run precision was estimated by analyzing 20 replicates of a calibration solution containing 20% methemoglobin (32 g/L). The mean result was 19.80% (SD = 0.64%, CV = 3.47%). We evaluated between-assay precision by determining the concentration of 29 calibration solutions with methemoglobin in the range of 10% to 100% (16 to 160 g/L) in four series of assays. The linear regression equation, relating the expected (y) and the measured (x) values, is \( y = 0.996x + 0.32 \) (r = 0.999).

For solutions with low concentrations of methemoglobin, we calculated the difference between the expected and the measured values for 10 samples in the range of 1% to 10% methemoglobin (1.6 to 16 g/L). The mean (±SD) error was 0.107% ± 0.077% (0.17 ± 0.12 g/L).

**Correlation with the Evelyn–Malloy method.** We compared the results obtained with our method (y) and an adaptation of the Evelyn–Malloy technique (x) for calibration solutions with various percentages of methemoglobin. For methemoglobin values >10%, correlation was good: \( y = 1.006x - 0.03 \) (r = 0.999). But lower concentrations showed no correlation because of the low precision of Evelyn–Malloy method. In the latter technic, the plot of absorbance at 630 nm vs concentration systematically deviated from the theoretical line of identity, in contrast to the good linearity of our method.

**Specificity.** Compounds that interfere with methemoglobin determination in the 630-nm region do not interfere with this method: methylene blue does not absorb near 400 nm, and the use of derivative spectrophotometry eliminated the influence of turbid samples (9, 10).

Bilirubin interferes slightly in hemoglobin determination by first-derivative spectrophotometry (13–15) but, owing to the large differences between the concentrations of these pigments in blood, we detected no perturbations from bilirubin concentrations as great as 100 mg/L.

**Normal blood controls.** The concentration of methemoglobin in 10 blood samples assayed as soon as they arrived in our laboratory was <1%. On the other hand, if the samples were left at room temperature and exposed to air for several hours, the methemoglobin percentage increased to as much as 4%.

**Discussion**

**Choice of spectrophotometric method.** The Soret region is very favorable for hemoglobin determination: absorptivity is high and the bandwidth at peak half-height is narrow, which gives high first-derivative values. But when a mixture of hemoglobins is present, it is difficult to separate the bands, which overlap considerably.

After the reduction of oxyhemoglobin, the carboxyhemoglobin peak (418 nm) can be separated from the hemoglobin peak (430 nm) by second- or fourth-derivative spectrophotometry (16, 17). However, this method fails for methemoglobin because reduction would convert both methemoglobin and oxyhemoglobin. To solve this problem, we decided to convert hemoglobin into oxyhemoglobin. Because the maxi-


mum of the oxy- and methemoglobin derivatives were very close (414 and 406 nm), it was difficult to obtain good results by using conventional "peak to peak" or "baseline" measurements with the first or second derivative (Figure 1). The zero-crossing-point first-derivative method is well suited to solve this problem (9, 11); at 414 nm, the first-derivative value of oxyhemoglobin curves is zero, whatever the concentration (Figure 2), so that the derivative value of an oxyhemoglobin–methemoglobin mixture at this wavelength is related to only the methemoglobin concentration.

To avoid systematic error, one must determine the exact wavelength at which the first-derivative spectrum for oxyhemoglobin reaches zero. Because this wavelength varies slightly, depending on the spectrophotometer's wavelength calibration and slit width and on the method used for smoothing derivative curves, we plotted the first-derivative
The spectrum of oxyhemoglobin in blood from a normal subject determines exactly its intersection with zero.

The changes in the slope of the hemoglobin spectrum were great in this spectral region, which gave steep slopes for the derivative curves and made it difficult to determine precisely the values on the graph. Then we used a dilution ($A_2$) of the normal blood ($A_1$) in the reference channel of the spectrophotometer; this decreased the slope of the derivative curve but did not change the measured value at the exact zero-crossing point (Figure 3).

**Preparation of solution.** Erythrocytes were easily lysed by dilution with a large volume of cold water. There is no need to eliminate cellular debris, because the effect of the slight turbidity they create is eliminated by using derivative spectrophotometry.

The exact volume of hydrogen peroxide solution necessary to convert all the hemoglobin into oxyhemoglobin was determined with blood samples from 10 normal subjects.

**Characteristics of the method.** The accuracy of the method described here is satisfactory, as shown by the linearity of the calibration curves. Its precision is good and the resulting values are well correlated with those obtained by the Evelyn–Malloy technique for measuring high concentrations of methemoglobin. But its main advantage lies in its sensitivity, easily determining low concentrations of methemoglobin.

In conclusion, our method meets the desired analytical criteria—especially sensitivity—for determining methemoglobin in blood. It is simple to perform and well adapted for use in emergency treatment of methemoglobinemia.

References