Measurement of Methemoglobin in Neonatal Samples Containing Fetal Hemoglobin

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Because of the potential for methemoglobinemia during nitric oxide therapy in newborns, methods are needed to accurately quantify methemoglobin (MetHb) in the presence of the high concentrations of fetal hemoglobin (Hb F), bilirubin, and lipids seen in these patients. Spectral differences between fetal and adult Hbs invalidate assumptions of conventional multiwavelength Hb photometry, so we evaluated an "overdetermined" system (Ciba-Corning Model 270), in which absorbances at seven wavelengths are measured to quantify four Hb derivatives. Adult and umbilical cord blood (Hb F 96%) samples were prepared to contain known MetHb fractions. Measured MetHb was linear in cord blood to ±15% MetHb. Within-run precision (CV) was <2.2% (n = 10) at each of seven MetHb fractions between 5% and 100%. Measured (y) and expected (x) MetHb fractions in cord blood were in good agreement (y = 1.0200x + 0.100, S_xy = 0). Added bilirubin (200 mg/L serum) and lipid (30 g/L) did not interfere. No significant differences were seen for adult and cord blood samples with identical MetHb fractions (P = 0.72), whereas a significant difference was noted with an exactly determined system (P = 0.0033). At clinically relevant MetHb fractions (<15%), a trend towards increased values in cord blood was noted with an exactly determined system (y = 1.0520x + 0.7600). We conclude that this overdetermined system measures MetHb accurately in samples from patients with large concentrations of Hb F.

Indexing Terms: nitric oxide/spectrophotometry/persistent pulmonary hypertension of the newborn

Inhaled nitric oxide (NO) is a promising but potentially toxic therapy for persistent pulmonary hypertension of the newborn (PPHN) (1-2). NO is a potent vasodilator that, because of its short half-life (3-6 s), acts locally on the pulmonary vasculature without producing systemic vasodilation. NO is a lipophilic gas that readily diffuses into vascular smooth muscle cells, where it binds to the heme moiety of soluble guanylate cyclase (5). The resulting activation of guanylate cyclase increases guanosine 3',5'-cyclic monophosphate (cGMP) (5), which decreases free Ca²⁺ concentrations (6, 7), and therefore produces vasodilation and decreased pressure in the pulmonary circulation.

Therapy with NO can produce toxic effects on blood as it passes through the pulmonary circulation. NO oxidizes the Fe(II) of erythrocyte hemoglobin (Hb) to the Fe(III) state to form methemoglobin (MetHb) (7). MetHb does not have the ability to reversibly bind oxygen, and increased MetHb impairs oxygen transport. This is an especially serious concern in PPHN, in which oxygenation is already impaired as a result of pulmonary hypoperfusion. Because MetHb fractions >5% may produce undesirable toxicity, NO therapy requires accurate monitoring of MetHb. Safety standards for NO exposure have been described elsewhere (8).

Conventional methods used to determine Hb and its derivatives rely on "exactly determined" spectrophotometric systems, in which the number of wavelengths employed equals the number of derivatives to be directly measured (9). While such methods are adequate for most samples, substances such as bilirubin, lipids (10), and Hb variants (9) may interfere. Newborn blood contains high concentrations of fetal hemoglobin (Hb F) (50-100%), which, because of its different globin chain composition, has slight spectral differences from adult hemoglobin (Hb A) (11). Hb F acts as an interfering substance on exactly determined spectrophotometers; this has been best documented as falsely increased carboxyhemoglobin (COHb) measurements in infants (12). The accuracy of measurement of MetHb in the presence of high Hb F has not been adequately studied. Newborn infants not infrequently develop increased bilirubin concentrations, and hospitalized infants frequently receive their nutrition through intravenous solutions supplemented with commercial fat emulsions. For these reasons, a method to measure MetHb in the presence of both high concentrations of Hb F and increased bilirubin or lipids is necessary.

We describe here our studies of a new multiwavelength Hb photometer, the Corning 270 CO-Oximeter (Ciba-Corning, Medfield, MA), that uses an overdetermined system employing seven wavelengths (557, 577, 597, 605, 624, 635, and 650 nm) to directly measure four Hb derivatives [oxyhemoglobin (Hb O₂), COHb, MetHb, and deoxyhemoglobin (HHb)]. The seven wavelengths were specifically chosen to minimize the spectral differences between Hb A and Hb F. It has been proposed that this type of system is more accurate in the presence of interfering substances and Hb F (9).

Materials and Methods

We collected into heparin-containing evacuated tubes blood from healthy men (<1.5% MetHb) and from fetal umbilical cords, following guidelines established at the University of Virginia Health Sciences Center. Hb F of the pooled cord blood sample was 95.9% of the
total hemoglobin concentration (ctHb) as determined by HPLC (American Medical Laboratories, Chantilly, VA).

We used the following methods for both adult and fetal blood to produce samples of each with known MetHb fractions. Heparinized blood (~30 mL, adult or cord) was divided equally into six 50-mL centrifuge tubes. In four tubes designated “0% MetHb,” cells were washed four times in 40 mL of isotonic saline (NaCl, 0.158 mol/L) with 10-min centrifugations at 110g. After the last centrifugation, the supernate was aspirated and the specimens were pooled. The volume was brought to ~20 mL with saline, and ctHb was determined with a Beckman (Irvine, CA) DU spectrophotometer by the standard cyanmethemoglobin method (13, 14). The ctHb was also determined on the Model 270 CO-Oximeter. The other two tubes of untreated whole blood were labeled “100% MetHb,” and the cells were washed once and then mixed with 10–15 mg of KNO₂ added to each tube to produce MetHb. The tubes were then incubated for 30 min in a 37°C water bath, followed by three washings (with saline as described above) to remove excess KNO₂. After the final centrifugation, the contents of the two incubated tubes were combined, and the volume was brought to ~10 mL with saline. The ctHb was determined as described above. The 0% MetHb and 100% MetHb samples were then brought to the same ctHb value by saline dilution of the sample with the greater ctHb.

Manual determination of MetHb fractions was performed on a Beckman DU spectrophotometer with the Evelyn–Malloy (KCN addition) method (15). This method is based on the absorbance maximum of MetHb at 630 nm, a property unique to this Hb derivative. The results documented that the 100% MetHb and 0% MetHb samples contained >99% and <2% MetHb, respectively. The samples were then used to produce samples with MetHb fractions of 0%, 5%, 10%, 15%, 25%, 50%, 75%, and 100%. The accuracy of the dilution for each of these samples was checked by measurements of MetHb by the Evelyn–Malloy method. For determination of absorption spectra, the samples were diluted 231-fold in deionized water.

MetHb was measured in all specimens with the Model 282 photometer (Instrumentation Laboratory, Lexington, MA) and the Model 270. Each specimen was analyzed 10 times on 1 day with the Model 270, from which a mean, SD, and CV were determined. Day-to-day imprecision could not be assessed with this material because of the instability of MetHb after 2–4 days.

To investigate the effects of bilirubinemia, we dissolved 20 mg of unconjugated bilirubin (Porphyron Products, Logan, UT) in 2 mL of 0.1 mol/L NaOH and added 0.1 mL of this stock solution to 5 mL of a solution with 60 g of bovine serum albumin per liter of saline. This created a “serum” with 200 mg/L unconjugated bilirubin and 60 g/L albumin. Equal portions of this solution and packed erythrocytes with a known MetHb fraction were mixed in triplicate, and each specimen was analyzed twice on the Model 270. A control sample contained erythrocytes in the same alkaline albumin solution but without bilirubin.

To investigate the effects of lipemia, we mixed 0.5 mL of a 200 g/L commercial fat emulsion (Intralipid; Baxter Laboratories, Deerfield, IL) with the bovine serum albumin solution (described above) to a total volume of 10 mL, for a total triglyceride concentration of 30 g/L. Equal parts of this “lipemic serum” and packed erythrocytes with a known MetHb fraction were mixed in triplicate, and each specimen was analyzed twice on the Model 270. A control sample included the same albumin solution and 0.5 mL of isotonic saline in place of the lipids.

Data comparing the measured vs nominal fractions of MetHb were analyzed by regression and analysis of covariance. Analysis of covariance of the results was carried out with the SAS general linear model program with a homogeneity of slopes model (SAS Institute, Cary, NC). The data obtained with the icteric and lipemic samples were assessed with unpaired t-tests.

Results
The first step in these studies was the preparation of samples with known fractions of MetHb. Representative spectra of these samples are shown in Fig. 1.

The overdetermined system implemented on the Model 270 yielded within-run imprecision (CV) of 0.06–2.16% and 0.12–1.05% for samples of cord and adult blood, respectively, with MetHb fractions in the range of 5–100%. The precision profiles for MetHb measured in adult and cord blood are shown in Fig. 2A. The production of MetHb decreased HbO₂ concentrations in the samples (which were essentially 100% HbO₂ because of exposure to room air during preparation), thus allowing the examination of HbO₂ measurements over a wide range of values. Within-run CVs for HbO₂ in samples of cord and adult blood were 0.08–
0.19% and 0.14–1.06%, respectively, at 30–99% oxyhemoglobin (Fig. 2B). MetHb results were linear for the cord blood samples over the range of 0–15% (r = 1.000).

As shown in Table 1, the measured (y) and expected (x) values obtained with the Model 270 for adult and cord blood MetHb fractions were in good agreement (y = 1.0200x + 0.1000, S_{yx} = 0) at the clinically relevant fractions of 0–15%. A similar comparison of the values obtained from the Model 282 yielded y = 1.0520x + 0.7600, S_{yx} = 0.0736. Adult and cord blood MetHb appeared to produce different responses on the Model 282: Analysis of covariance revealed a significant difference (P = 0.0033) between the slopes of measured vs expected values for adult and umbilical cord blood samples. No significant differences were noted between adult and cord blood on the Model 270 (P = 0.7247).

The measured MetHb fraction of a cord blood pool prior to addition of bilirubin or Intralipid was 0.82% ± 0.08% (n = 3); values were unchanged after these additions (0.82% ± 0.20% and 0.80% ± 0.09%, respectively). Unpaired t-tests of these data revealed no significant differences (P > 0.05).

The new instrument yielded progressively more negative HHb values for all cord blood samples with MetHb >5% (-2.5% HHb at 5% MetHb to -16.9% HHb at 100% MetHb) with corresponding numerically equivalent increases in COHb (1.6% COHb at 5% MetHb to 18.1% COHb at 100% MetHb). The falsely increased COHb fractions in fetal blood measured by the exactly determined multiwavelength Hb photometer that have been previously described (12) were seen on the older Model 282.

**Discussion**

The present studies document the linearity, precision, and recovery of fetal MetHb measurements with the overdetermined system of the Model 270 CO-Oximeter. As MetHb increased to >25% of the tHb, the agreement with expected values decreased, a phenomenon seen with both the exactly determined and the overdetermined systems. As these MetHb fractions are well above those to be expected in trials of NO therapy, the analysis of data presented here was performed only for MetHb fractions ≤15%.

The use of KNO₂ to produce intraerythrocyte MetHb in our experiments also produces NO₂MetHb, as described by Van Assendelft and Zijlstra (16). This Hb derivative has slight spectral differences from MetHb, and must be removed by carefully washing the erythrocytes multiple times with isotonic saline, which effectively converts all NO₂MetHb to MetHb (16). A maximal absorbance peak at 630 nm in the 100% MetHb sample indicated the complete conversion to MetHb. A peak at 625 nm would be expected in the presence of NO₂MetHb (16).

Although formal evaluation of the performance of the IL Model 282 as a prototype of an exactly determined system for measurement of MetHb in cord blood was not an objective of this study, our limited comparison measurements suggest that this instrument produced falsely increased values at clinically relevant MetHb fractions. Measurements of the 5% MetHb sample by

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* Cord blood measurements obtained by the Model 282 represent the means of two determinations on consecutive days.

**Fig. 2.** Within-run precision profiles (n = 10) for MetHb (A) and HbO₂ (B) measured in adult (dashed line) and cord (solid line) blood on the Model 270 CO-Oximeter. Samples were prepared as described in Materials and Methods.

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Table 1. MetHb results in adult and cord blood samples.

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* Cord blood measurements obtained by the Model 282 represent the means of two determinations on consecutive days.
the Model 282 gave results of ~6% MetHb, or ~20% greater than expected. Although this discrepancy in measurement would not alter the course of treatment in most situations, falsely increased fractions near a “threshold” value (e.g., 5%) might lead to cessation of NO therapy. Congenital methemoglobinemia may present with MetHb fractions as high as 25–30% (17), so the accuracy of measurement of MetHb fractions at these greatly increased values (which were measured as 8% lower than actual values in these studies) is still adequate for making this diagnosis. Further studies will be required to document the performance of the various multiwavelength Hb photometry systems if they are to be used to measure MetHb in cord blood.

Zijlstra and others have devised a useful method to correct for the spectral differences between Hb A and Hb F (11). Unfortunately, this requires prior knowledge of Hb F concentrations, which can be impractical for the monitoring of NO therapy, as changes in MetHb may occur rapidly in infants. Overdetermined systems that exploit the similarities of the spectra of Hbs A and F (and their derivatives) offer a simpler alternative.

Bilirubinemia (200 mg/L) and lipemia (30 g/L) did not interfere with the Model 270 measurements of MetHb fractions in the presence of high concentrations of Hb F. Interference from bilirubin would not be expected with this instrument, because bilirubin exhibits negligible absorbance, even at the lowest wavelength analyzed on the Model 270 (557 nm) (18). The lack of interference from lipids is another attractive feature of overdetermined systems.

The progressive changes in measured HHb and COHb that were seen with increasing MetHb suggest a flaw in the multicomponent analysis used in the Model 270. As the samples were prepared in such a manner that essentially all of the active Hb was converted to HbO2 and the MetHb fractions were confirmed by the KCN addition method, these results indicate an obligation overestimation of the Hb oxygen saturation [cHbO2/(cHbO2 + cHHb)] as well as COHb fractions. In the cord blood samples, the sum of the HbO2 and MetHb fractions remained 100% at all MetHb fractions. Although the errors in oxygen saturation and COHb are not likely to be clinically significant, this limitation must be recognized. HHb is not reported on the IL 282, but it too must be incorrectly measured, as the sum of the reported Hb species (HbO2, COHb, and MetHb) progressively increases to >100% as the MetHb fractions increase.

In conclusion, the Ciba-Corning Model 270 CO-Oximeter uses an overdetermined spectrophotometric method to more accurately measure Hb derivatives in the presence of interfering substances and Hb variants. Our study supports this finding, while suggesting possible inaccuracies with the more commonly used exactly determined systems.

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References