Improved Quantitative Apt Test for Detecting Fetal Hemoglobin in Bloody Stools of Newborns
Ning Liu, Alan H. B. Wu, and Shan S. Wong

We devised an improved spectrophotometric method, based on the differential susceptibility of various hemoglobins to alkaline denaturation, to measure the percentage of fetal hemoglobin (HbF) in bloody meconium so as to determine the source of the blood. The oxyhemoglobin of a sample was spectrophotometrically scanned from 450 to 650 nm before and after the addition of sodium hydroxide. With Allen baseline correction, the ratio of absorbance at 576 nm was linearly proportional to the percentage of HbF in the specimen as described by the equation: ratio = 0.01 (%HbF) + 0.0045 (t = 0.9971, S_y|x = 0.047). Preliminary studies on samples containing 10% or 80% HbF revealed that the method was fairly precise. The accuracy of this test was verified by comparing the results (y) with those obtained by radial immunodiffusion (x): y = 0.997x + 2.93 (r = 0.91). This preliminary study demonstrates that the modified test is simple, fast, and free from bilirubin interference. It should be useful for the diagnosis of gastrointestinal bleeding of the newborn on a stat basis.

Indexing Terms: meconium • gastrointestinal bleeding • hemoglobin variants • pediatric chemistry

In the differential diagnosis of gastrointestinal bleeding of the newborn, the bloody discharge is analyzed to distinguish whether the hemoglobin (Hb) is fetal in origin or derived from the mother. On the basis of the different susceptibilities of HbA and HbF to alkaline denaturation, Apt and Downey developed a test for detecting HbF (1). In this procedure, sodium hydroxide is added to the test solution to convert oxyhemoglobin to hematin. For normal adult blood, which usually contains <7% HbF, the pink color (oxyhemoglobin) quickly changes to brownish-yellow (alkaline hematin) within 2 min. If fetal blood, which usually contains 50%-90% HbF, is present, the pink color persists. Although this procedure has been recommended and adopted in numerous laboratories (2), such subjective visual judgment of color change occasionally leads to misinterpretation or inconclusive results, particularly when only a small amount of blood is present in the stool specimen. Moreover, because of stool color interference, grossly bloody specimens are required for the test; however, they are usually unavailable in clinical situations. Thus, a more sensitive and accurate method is needed to estimate the HbF percentage in a small amount of the sample.

Quantification of HbF percentage can be accomplished by various methods. Radial immunodiffusion and electrophoresis are most commonly used in clinical laboratories. However, Hb electrophoresis is, at best, only semiquantitative; neither method is sensitive; and both suffer from interferences by meconium, are time-consuming, and are labor intensive. In spectrophotometric measurement of HbF, first developed by Brinkman and Jonxis in 1985 (3) and later modified by Singer et al. (4), fetal oxyhemoglobin was quantified at 540 nm. We have now applied this spectrophotometric measurement to modify the Apt test and report here our preliminary results with this method.

Materials and Methods

Blood samples containing normal adult hemoglobin (HbA) or HbF (cord blood) were obtained from Hermann Hospital Clinical Laboratory (Houston, TX). All the samples were analyzed for total Hb content with an IL 282 Co-Oximeter (Instrumentation Laboratory, Lexington, MA), and for the presence of HbF by electrophoresis (Paragon hemoglobin electrophoresis kit; Beckman, Brea, CA). Radial immunodiffusion (Helena Laboratory, Beaumont, TX) was used to determine the concentration of HbF. The percentages of HbF were calculated from the measurements of HbF and total Hb values. Meconium was obtained from normal newborns at the Hermann Hospital nursery.

For accuracy studies, we mixed known amounts of HbF with adult blood and meconium to provide a range of concentrations (shown later in Figure 4). These preparations (about 20 mg) were mixed with 2 to 10 mL of deionized water to provide a final Hb concentration of ~10 mg/L. The suspension was centrifuged at 500 × g for 10 min to remove insoluble debris. If the supernate remained contaminated, the solution was further clarified by being forced through a 0.45-μm (pore size) syringe filter. The filtrate was scanned spectrophotometrically with a Cary 118 spectrometer (Varian Instruments, Sunnyvale, CA) from 450 to 650 nm. The absorbance at 576 nm was measured with Allen baseline correction. Sodium hydroxide (0.25 mol/L) was added directly to the sample in the cuvette (1:5 by vol) to give a final concentration of 41.7 mmol/L. After 2 min the solution was scanned again as before. The ratio of absorbances at 576 nm, after and before alkaline denaturation, was calculated after correction for dilution.

To compare the Apt test with the modified procedure,
we mixed adult and cord blood of known Hb compositions and concentrations with meconium to generate samples containing 10% to 80% of total Hb each with 80% to 10% as HbF, respectively. These samples were analyzed spectrophotometrically as described above. For the Apt test, the color of the solution after the 2-min incubation was evaluated by an individual who was not informed of the concentration of HbF.

To determine the reproducibility of the procedure, we repeatedly assayed, either in one setting (within-run) or in several days (between-run), meconium preparations containing 10% or 80% HbF. The percentages of HbF were calculated by using the equation obtained in the accuracy studies.

Results

Apt test. The results obtained by visual judgment of color change 2 min after alkali denaturation are shown in Table 1. In samples with 40% or more total Hb, the typical color change from pink to yellow or yellow-brown could be observed. However, in meconium containing 20% or less total Hb, the meconium color was predominant and the typical color change could not be accurately determined. Irrespective of total Hb content, the color of samples containing 40% or 60% HbF after alkaline denaturation depended on subjective judgment. The interpretation of the results may be inconclusive.

Linearity of modified Apt test. The absorbance spectra of oxyhemoglobin, alkaline hematin, and meconium are shown in Figure 1. Oxyhemoglobin absorbs maximally at 412, 540, and 576 nm (5). Although meconium can strongly interfere with the spectrum at 412 nm, its influence becomes much smaller around 540 and 576 nm and can be eliminated with the Allen baseline correction. Also, the alkaline hematin has no absorbance above 500 nm. Thus the absorbance at 576 nm before the addition of sodium hydroxide can be used to represent the total oxyhemoglobin, whereas its absorbance after the addition of sodium hydroxide represents oxyhemoglobin F. Figure 2 shows that the absorbance intensity at 576 nm before and after alkaline denaturation reflects the concentration of HbF. When the ratios of absorbance after alkaline denaturation to that before denaturation are plotted against the percentages of HbF determined by radial immunodiffusion, a straight line is obtained (Figure 3). Linear regression analysis yielded the equation: absorbance ratio = 0.0107 (%F) - 0.0045 (r = 0.9971; S_{xy} = 0.0471). Meconium, constituting from 10% to 80% (by wt.) of the mixed blood and stool, did not interfere (Table 2). The absorbance ratios at each HbF percentage varied by <14%, which is within the experimental error of the procedure.

Accuracy. To assess the accuracy of the modified method, we separately mixed with meconium 10 cord blood and 10 adult blood samples with known HbF percentages (determined by radial immunodiffusion) and analyzed the resulting mixtures. The results are plotted in Figure 4. Regression analysis gave the line: y = 0.997x + 2.93 (r = 0.91).

Reproducibility. The precision of the method was investigated by using meconium preparations containing either 10% or 80% HbF. Due to lack of material, only five replicate assays could be performed. The estimated within-run CVs were 15% and 5.3% for HbF of 10% and 80%, respectively; the corresponding between-run CVs were 14% and 3.1%.

Discussion

In diagnosing a newborn for lower gastrointestinal tract bleeding, a relatively rapid and accurate assay to rule out swallowed maternal blood is necessary. Usually the general condition of the newborn and the mother's
Table 2. Absorbance Ratios for Different Hb Contents

<table>
<thead>
<tr>
<th>HbF, %</th>
<th>10%</th>
<th>20%</th>
<th>40%</th>
<th>60%</th>
<th>80%</th>
<th>80%</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0.08</td>
<td>0.10</td>
<td>0.12</td>
<td>0.08</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>0.18</td>
<td>0.24</td>
<td>0.25</td>
<td>0.21</td>
<td>0.18</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>0.38</td>
<td>0.52</td>
<td>0.46</td>
<td>0.34</td>
<td>0.38</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>0.67</td>
<td>0.68</td>
<td>0.70</td>
<td>0.47</td>
<td>0.48</td>
<td></td>
</tr>
<tr>
<td>80</td>
<td>0.95</td>
<td>0.87</td>
<td>0.93</td>
<td>0.62</td>
<td>0.90</td>
<td></td>
</tr>
</tbody>
</table>

*Peak height at A_{578} nm, after alkaline denaturation.

Laboring process may provide some useful information with regard to the blood origin. However, a clinical test should provide more definitive information. The original Apt test, being based on visual interpretation, is subjective. In most instances, inclusive results are obtained (Table 1). With a spectrophotometer, HbF may be quantified at a wavelength away from the absorbances of contaminants (see Figure 1 and Table 2). This process increases the sensitivity and accuracy of the test and is reproducible over a wide range of HbF and total Hb contents (Figure 1 and Table 2). In addition, the process is simple and takes only ~30 min.

In applying this method to clinical samples, we suggest that the Apt test be performed first. When the visual result is inclusive or when a quantitative result is desired, the modified spectrophotometric test may be used. Mix the sample (~500 mg) with 5 mL of deionized water and follow the experimental procedure outlined in Materials and Methods. Analyze the data obtained by using the following equation (derived from the accuracy experiment): \% HbF = 95 \times (A_2/A_1), where A_2 is the absorbance after denaturation and A_1 is the absorbance before denaturation.

The sensitivity of the spectrophotometric measurement yields a lower limit of detection of 1 mg/L Hb; the

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Fig. 1. Spectrophotometric scan of oxyhemoglobin before (---) and after (----) alkaline denaturation

Fig. 2. Spectrophotometric scan of oxyhemoglobin before (---) and after (----) alkaline denaturation

Fig. 3. Plot of A_2/A_1 in Fig. 2 vs HbF%

Fig. 4. Plot of HbF% determined by radial immunodiffusion vs that determined by the modified Apt test

Ten samples, each containing HbA and different concentrations of HbF, were assayed by both methods and compared.
upper range of linearity is \( \sim 2000 \) mg/L. Thus this method may be performed with meconium samples containing HbF as little as 10% of total Hb by weight, for which the original color of the specimen may be brownish-green. Samples with relatively high Hb content may need to be diluted. To obtain the proper dilution, use a freshly made hemolysate containing 20 \( \mu \)L of blood in 5 mL of water for comparison.

There are no commercial quality-control samples available for assay of these specimens. However, control specimens can be easily prepared by lysing fresh blood cells in deionized water to generate a Hb solution of \( \sim 10 \) mg/L. A positive blood specimen containing HbF should also be used. These solutions can be aliquoted into 1.5-mL fractions and, stored frozen or refrigerated, should be stable for at least 6 months. Stored at 4 °C, HbF decreases by \( \sim 10\% \) after 1 year.

An HbF content >50% probably indicates that the blood is fetal in origin. Blood with HbF <10% definitely comes from the mother. Obviously, this procedure cannot distinguish fetal blood from certain hemoglobinopathies such as hereditary persistent HbF.

An average error for this method is \( \sim 10\% \) (Table 2). An experimental error of this magnitude will not affect the deduction of source of blood in the meconium. Consequently, the spectrophotometric modification of the Apt test can unambiguously distinguish the origin of blood in a newborn's discharge.

References

HemoCue \( \beta \)-Glucose Photometer Evaluated for Use in a Neonatal Intensive Care Unit
Elizabeth Vadasdi\(^1\) and Ellis Jacobs\(^1,2,3\)

We evaluated the HemoCue \( \beta \)-Glucose Photometer system for use in our neonatal intensive care unit by assaying 178 heparinized whole-blood samples obtained by heel stick. The required sample size is 5 \( \mu \)L. Plasma glucose was analyzed by a glucose oxidase/oxygen electrode methodology. Across the glucose range of 1.28–21.87 mmol/L, the regression slope was 0.976 (\( r = 0.976, S_{yx} = 0.475 \)). For samples with hematocrit \( \leq 0.30 \), the regression slope was 0.981 (\( r = 0.950, S_{yx} = 0.415 \)); for hematocrit of 0.31–0.49, the regression slope was 0.984 (\( r = 0.972, S_{yx} = 0.508 \)); and for hematocrit \( \geq 0.50 \), the regression slope was 0.959 (\( r = 0.998, S_{yx} = 0.394 \)).

Human whole blood, bovine whole blood, and bovine serum-based quality-control materials were studied. Except for assays of the low-concentration human control material, the total CV was <3.5%. The accuracy and precision of the HemoCue system were comparable with those of conventional laboratory instrumentation.

Indexing Terms: reflectance photometry • point-of-care testing • hematocrit • critical care medicine • pediatric chemistry

\(^1\) Center for Clinical Laboratories, The Mount Sinai Hospital, and \(^2\) Department of Pathology, Mount Sinai School of Medicine, New York, NY 10029-6574.
\(^3\) Address correspondence to this author at: The Mount Sinai Medical Center, STAT Laboratories, Box 1519, One Gustave L. Levy Place, New York, NY 10029-6574. Fax 212-876-0651.

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For the rapid assessment of glucose homeostasis, reagent strips impregnated with glucose oxidase have been available since the 1980s, and portable reflectance meters based on this principle have been marketed since 1970. By the 1980s, bedside capillary glucose monitoring had become a standard of practice even though several authors expressed concerns with the accuracy of the measurement (1–3). New and modified solid-phase reagent strips/meter systems were developed for utilization with the latest reflectance technology (e.g., Glucometer, Ames; One Touch, Lifescan; Accu Chek II, Boehringer Mannheim Diagnostics) and electrochemical technology (Satellite G, MediSenese) (4–6). All these developments improved the accuracy of measurement, but none fully eliminated the effects of extreme hematocrit values on glucose determination in whole-blood samples.

In clinical practice, dry-reagent strip methods are widely used to screen for abnormal glucose concentration, most frequently for identifying neonatal hypoglycemia (7, 8). Several reports have emphasized (7–9) that, although dry-reagent strip technology is useful for screening glucose in neonates, it cannot be relied upon for monitoring glucose homeostasis. Thus, before any therapeutic intervention, confirmation by a conventional laboratory method is necessary.

In our previous investigation of the influence of he-