Spectrophotometry of Occult Blood in Feces

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This method for measuring fecal occult blood is based on the heme-catalyzed oxidation of tetramethylbenzidine by H₂O₂. An aliquot of heated stool homogenate is mixed with acetic acid to chemically separate heme from globin. The heme is extracted into ethyl acetate and reacted with the reagent and H₂O₂ to produce a green oxidation product. The reaction is followed kinetically for 30 to 60 s at 660 nm. Asec is linearly related to the amount of hemoglobin. The lower limit of detection is 1 to 2 mg of hemoglobin per gram (wet weight) of feces. Within-day precision (CV) of the analysis for hemoglobin added to stool specimens (4 to 30 mg/g) ranged from 2.3 to 7.6%, between-day CV from 2.1 to 8.1%. Analytical recovery of hemoglobin added to fecal specimens (4 to 30 mg/g) ranged from 86.7 to 106.2%. Of the substances known to interfere with conventional dyes-oxidation tests for fecal occult blood, only myoglobin and ascorbic acid interfere with hemoglobin quantification by our procedure. The test is fast, inexpensive, and easy to perform, and involves equipment available in hospital laboratories.

Additional Keyphrases: heme as a catalyst · tetramethylbenzidine · kinetic assay · cancer

Colorectal cancer is one of the most common cancers in the United States; more than 120 000 new cases are diagnosed yearly (1). The current mortality rate of this disease exceeds 50%, and the five-year survival rate of colorectal cancer patients is directly correlated with the pathologic stage of the cancer at diagnosis (1, 2). The most widely used marker for early detection of colorectal cancer is occult blood in the feces, for which there are several commercially available tests. These tests typically involve applying a sample of stool specimen to a guaiac-impregnated filter paper. Occult blood hemoglobin in the feces catalyzes the oxidation of guaiac by hydrogen peroxide to produce a blue color. This reaction depends on the pseudoperoxidase activity of the heme moiety of hemoglobin (3). Such tests are fast and economical but are subject to false-positive and false-negative reactions (4–6) and are only qualitative. This complicates distinguishing normal from increased fecal blood loss, particularly because these tests differ significantly in their sensitivities to hemoglobin (6).

Recently, Jaffe and Zierdt (7) described an occult blood assay in which the heme component of stool hemoglobin is extracted and reacted with tetramethylbenzidine (TMB) in the presence of hydrogen peroxide to yield a green oxidation product. This procedure eliminates most interferences that affect tests for occult blood that depend on dye-oxidation. However, their assay is only qualitative.

Surprisingly little effort has been directed toward development of quantitative assays for occult blood in feces. Currently available methods involve either radioactive tracers (6, 8) or production of antibodies to hemoglobin (9, 10). Radioisotopic procedures for quantification of fecal occult blood are valuable for investigative purposes but are too cumbersome for routine use in most hospital laboratories. Immunohemoglobin antibodies are more amenable to routine use but are subject to interferences related to loss of antigenic activity of hemoglobin by bacterial action in feces as well as adsorption problems caused by fecal particulate matter.

Here we describe a simple, rapid, quantitative procedure for determining fecal occult blood. This procedure is not subject to most of the interferences that affect other dye-oxidation-based tests for occult blood that are currently in use, and it involves reagents and equipment readily available in clinical laboratories.

Materials and Methods

Apparatus. We used a Model DB spectrophotometer (Beckman Instruments, Inc., Fullerton, CA 92636) with a Recordall 5000 Recorder (Fisher Scientific Co., Pittsburgh, PA 15219). Hemoglobin content of aqueous human erythrocyte standards was determined with a Model S Plus Coulter Counter (Coulter Electronics, Hialeah, FL 33010).

Reagents and standards. Prepare a 60 mmol/L stock solution of TMB dye by dissolving 2.88 g of 3,3′,5,5′-tetramethylbenzidine (Aldrich Chemical Co., Inc., Milwaukee, WI 53201) in 200 mL of a solvent mixture consisting of glacial acetic acid/distilled water/absolute ethanol (20/30/50 by vol). To stabilize the TMB dye solution, add 1.55 g of histidine and 50 mg of 2,4-dinitrophenylhydrazine to give final concentrations of 50 and 1 mmol/L, respectively. Stored at 4 °C in a brown-glass bottle, this stock dye solution is stable for at least three months.

Prepare a 0.6 mmol/L TMB working solution by diluting the stock solution 100-fold in the acetic acid/H₂O/ethanol solvent mixture. The working solution is stable in a brown-glass bottle at 4 °C for at least two weeks.

Determine the hemoglobin (Hb) content of packed human erythrocytes and suspend appropriate aliquots of the erythrocytes in distilled water to make a 2.0 g/L hemoglobin standard. This standard is stable for two weeks at 4 °C. Crystaline horseradish peroxidase (EC 1.11.1.7), beef liver catalase (EC 1.11.1.6), and equine muscle myoglobin were obtained from Sigma Chemical Co., St. Louis, MO 63178.

All chemicals used were reagent grade.

Procedures. Prepare a homogenate of wet feces (5 g/100 mL of distilled water) in a Waring Blender operated at full speed for 30 s. Take the fecal specimen from several areas of the stool, to minimize error due to inhomogeneous distribution of blood in the feces (11). Alternatively, sampling error can be eliminated by homogenizing the total weighed specimen in a small, known volume of water, followed by removal of an aliquot equivalent to 5 g of feces for preparation of the 5 g/100 mL homogenate. Pipet 0.5 mL of homogenate (25 mg of feces) into a 16 × 125 mm screw-capped test tube and place it in a boiling water bath for 10 min to inactivate plant peroxidases. Remove the tube from the bath, add 3.0 mL of acetic acid/water (30/70 by vol), mix,
and let stand for 2 min. Add 4.5 mL of ethyl acetate, shake gently for 2 min, and centrifuge (3 min, 2000 \( \times \) g) to separate the aqueous and organic layers. Pipet duplicate 1.0-mL aliquots of the ethyl acetate (upper) layer into separate glass cuvetts (1.0-cm pathlength). Pipet 1.0 mL of TMB working solution into each cuvet and mix thoroughly. Place one cuvet into the reference side, the other into the sample side of a dual-beam spectrophotometer set at 660 nm. Adjust the absorbance to zero. Pipet 0.5 mL of distilled water into the reference cuvet and mix thoroughly. Initiate the oxidation reaction by pipetting 0.5 mL of a 30 mL/L solution of hydrogen peroxide in water into the sample cuvet. Mix the cuvet contents vigorously with the pipet. Follow the increase in absorbance (\( \Delta A_{660} \)) for at least 1 min. (A strip-chart recorder is very useful for this step.) Although not as convenient as a double-beam instrument, a single-beam spectrophotometer can be used as an alternative.

Standard curves are constructed by using aqueous erythrocyte hemoglobin solutions subjected to the above assay procedure, excluding the homogenization step. Two standard curves, one each for \( \Delta A_{660} \) at 30 and 60 s, are constructed by using 0.1–1.0 mg hemoglobin standards. The hemoglobin content of fecal specimens is determined from each standard curve and averaged. The use of \( \Delta A_{660} \) at both 30 and 60 s serves as a check on the linearity of the reaction, but is not necessary if the reaction linearity is monitored with a strip-chart recorder.

Calculate fecal Hb content as follows:

\[
\text{mg Hb} = \frac{(\text{mg Hb (30 s)} + \text{mg Hb (60 s)})}{2} \times 0.025 \text{ g feces}
\]

The hemoglobin standard curve (0.1–1.0 mg of Hb) corresponds to a fecal hemoglobin content of 4 to 40 mg/g of feces. Specimens containing hemoglobin in excess of 40 mg/g of feces may be diluted with additional distilled water and the determined result multiplied by the appropriate dilution factor.

**Results**

Figure 1 shows the absorption spectrum obtained when an aqueous solution of hemoglobin is mixed with acetic acid, extracted with ethyl acetate, and the extract reacted with tetramethylbenzidine and hydrogen peroxide. There are two absorption maxima, at 660 and 450 nm. The former peak is seen only after the \( H_2O_2 \) is added. The latter peak, which is present with or without \( H_2O_2 \), is intensified in the presence of the oxidizing agent. Figure 2 illustrates the absorption spectrum produced when a hemoglobin-supplemented stool specimen was treated as above. Again, the addition of hydrogen peroxide intensified the broad absorbance band at 400–500 nm, and a new band appears at 660 nm. Note the low background absorbance in the region of the second peak in the absence of \( H_2O_2 \). In all instances we observed that the height of the peak at 660 nm increased linearly with time after the addition of \( H_2O_2 \). We conclude that the increase in \( \Delta A_{660} \) at 30 and 60 s after addition of \( H_2O_2 \) is most useful for determining the amount of hemoglobin in feces from the viewpoints of speed and magnitude of change in absorbance.

Figure 3 illustrates the results of analytical recovery studies, in which hemoglobin was added in amounts from 4 to 40 mg/g of feces (0.1 to 1.0 mg of Hb per 0.5 mL of stool homogenate) to stool specimens containing no detectable hemoglobin and the results were compared with aqueous hemoglobin standards. Both standards and supplemented hemoglobin specimens exhibited excellent linearity (\( r = 0.997 \) to 0.999) over the range of hemoglobin concentration studied. At the 30- and 60-s reaction times, the regression lines for the supplemented stool specimens and the aqueous standards could be considered to be superimposable. The range over which the 60-s reaction time is linear (4–30 mg/g) is narrower than that (4–40 mg/g) for the 30-s \( \Delta A_{660} \).

The limit of detection of hemoglobin was about 2 mg/g of feces, which may be decreased to 1 mg/g of feces if 5 g of feces is homogenized in 50 mL of water instead of the usual 100 mL. We determined the precision of the method by adding hemoglobin (4, 10, and 30 mg/g of feces) to stool specimens in which no hemoglobin was detectable and subsequently repeatedly analyzing samples of these specimens. Within-day precision was determined by assaying each specimen 10 times in one day. Between-day variability was determined by assaying one specimen at each concentration daily for 10
days. The analytical recovery of hemoglobin from feces was determined by reading the hemoglobin concentration of supplemented stools from standard curves prepared by use of aqueous erythrocyte standards. These results are shown in Table 1.

Table 2 illustrates the effect on our assay of adding various substances that have been reported to interfere with occult blood detection.

To assess the clinical applicability of our assay, we assayed by our quantitative procedure 36 fecal specimens that were positive for occult blood by the "Hemoccult" slide test (Smith-Kline Diagnostics, Sunnyvale, CA 94086). The amount of hemoglobin detected ranged from 2 to 113 mg/g of feces. Of 100 different specimens negative for occult blood by the Hemoccult test, 12 contained detectable amounts of hemoglobin, ranging from 2 to 14 mg/g of feces, by our method. This indicates a variable sensitivity of the Hemoccult slide test to hemoglobin in patients' fecal specimens. (All Hemoccult slide tests for fecal occult blood were performed on unhomogenized fecal samples according to the kit instructions.)

Our procedure was also compared with another recently developed quantitative fecal hemoglobin assay (S. Schwartz, unpublished data) in which hemoglobin is converted to porphyrin and the resulting fluorescence is measured after extensive purification. Forty fecal specimens assayed by our procedure (γ) as well as the more laborious fluorometric method (α) gave the following regression equation: γ = 1.38x - 0.002 mg Hb/g of feces (r = 0.966). Hemoglobin content of these specimens ranged from 1.3 to 113 mg/g by our procedure and from 0.41 to 91.5 mg/g by the fluorometric technique. These data show a good correlation between these two quantitative procedures, although our method tends to give higher results for fecal hemoglobin than does the fluorometric technique. Inhomogeneous distribution of blood in the feces may partly account for this difference, there being a greater probability of sampling error in the fluorometric assay, given the small aliquot of stool analyzed (8 mg).

Discussion

Our assay for hemoglobin in feces, which involves the oxidation of tetramethylbenzidine in the presence of hydrogen peroxide and peroxidase activity to a green-colored product, appears to be reliable. The reaction depends on the peroxidase activity of the heme moiety of hemoglobin. Jaffe and Zierdt (7) separated heme from globin by treating feces with acetic acid, then extracting the heme into ethyl acetate and reacting it with TMB and H2O2. We have modified this qualitative procedure so that it is possible to actually measure fecal blood loss with equipment and reagents available to most clinical laboratories.

In contrast to the false-negative results reported after storage of fecal specimens when assayed by the Hemoccult procedure (8), we were unable to detect deterioration of unhomogenized or homogenized specimens stored for as long as two weeks at 4°C. Furthermore, although many substances reportedly interfere with the commercially available dye-oxidation (guaiac-slide) tests for occult blood in feces—including plant peroxidases, catalases, myoglobin, dietary iron supplements, halogens, cimetidine, and ascorbic acid (3, 5, 7, 12–15)—our assay was not affected by ferrous sulfate, ferric sulfate, methyl iodine, or cimetidine in concentrations of 10 mg/g of feces. When 10 mg of horseradish peroxidase or beef-liver catalase was added to feces per gram, no increase in apparent hemoglobin was observed, and only a negligible effect was noted at 20 mg/g. Of the compounds we tested, only myoglobin gave a significant increase in the amount of apparent hemoglobin, its ΔA660 being almost equal to that of hemoglobin.

Glutathione and penicillamine, two sulfhydryl reagents that theoretically can inhibit dye-oxidation-based tests for stool occult blood, caused no interference with our procedure when added to feces in concentrations of 10 mg/g. This was also the case with ascorbic acid at 4 mg/g of stool, but at 10 mg/g this reducing agent decreased the apparent hemoglobin content of supplemented stool samples (20 mg Hb/g of feces) by 60%.

The significance of the interference of myoglobin (from meat) and ascorbic acid with our assay in vivo is not clear, but ingestion of large amounts of these substances should probably be restricted in patients providing stool specimens for quantitative hemoglobin determination.

Unlike benzidine, which has been used to detect fecal occult blood loss, TMB is believed to be noncarcinogenic (7) and shows considerable promise as a suitable replacement for benzidine in laboratory procedures. However, general precautions should still be followed when working with TMB, such as performing the assay in a well-ventilated room and avoiding skin contact with the dye reagent.

Various upper limits for normal fecal blood loss have been proposed (16–18). These range from 1 to 5 mL of blood per day (about 1.4 to 7.0 mg of Hb per gram wet weight of feces). In our experience, in the presence of hemorrhagic gut lesions this excretion rises abruptly above 3.2 mg of hemo-

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**Table 1. Precision and Recovery Data for Hemoglobin in Stool**

<table>
<thead>
<tr>
<th>30-s reaction: added Hb, mg/g feces</th>
<th>60-s reaction: added Hb, mg/g feces</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean, mg/g</td>
<td>4.0</td>
<td>4.0</td>
</tr>
<tr>
<td>SD, mg/g</td>
<td>0.22</td>
<td>0.23</td>
</tr>
<tr>
<td>CV, %</td>
<td>5.2</td>
<td>5.5</td>
</tr>
<tr>
<td>% recovery (n = 20)</td>
<td>9.6</td>
<td>9.3</td>
</tr>
<tr>
<td>Mean</td>
<td>106.2</td>
<td>97.8</td>
</tr>
<tr>
<td>SD, %</td>
<td>2.1</td>
<td>3.1</td>
</tr>
</tbody>
</table>

**Table 2. Compounds Tested for Possible Interference with the Quantitative Fecal-Hemoglobin Assay**

<table>
<thead>
<tr>
<th>Added compound</th>
<th>&quot;Hb&quot; detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A</td>
<td></td>
</tr>
<tr>
<td>Ferric sulfate</td>
<td>10</td>
</tr>
<tr>
<td>Ferrous sulfate</td>
<td>10</td>
</tr>
<tr>
<td>CH3I</td>
<td>10</td>
</tr>
<tr>
<td>Cimetine</td>
<td>10</td>
</tr>
<tr>
<td>Horseradish peroxidase</td>
<td>10</td>
</tr>
<tr>
<td>Catalase</td>
<td>10</td>
</tr>
<tr>
<td>Myoglobin</td>
<td>10</td>
</tr>
<tr>
<td>Group B</td>
<td>9.6</td>
</tr>
<tr>
<td>Glutathione</td>
<td>10</td>
</tr>
<tr>
<td>Penicillamine</td>
<td>10</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>4</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>10</td>
</tr>
</tbody>
</table>

Group A = compounds checked for false-positive reaction (added to stool homogenates previously found to contain no hemoglobin); Group B = compounds checked for false-negative reaction (added to stool homogenates containing 20 mg Hb/g stool).
globin per gram of feces (D. A. Ahlquist, personal communication). Fecal occult blood loss, expressed as mg Hb/g wet weight of feces in our procedure, can be easily converted to mg Hb/day or mL blood/day if the weight of the total fecal specimen, the time period of collection, and the blood Hb and hematocrit are obtained. Our quantitative assay, with its detection limit of 1–2 mg of hemoglobin per gram of feces, should prove valuable in discriminating between normal, mildly, and grossly abnormal losses of blood in feces. This may be important in the early detection of colorectal cancer.

References