Free Erythrocyte Porphyrins in the Detection of Undue Absorption of Pb and of Fe Deficiency

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A fluorometric assay for free erythrocyte porphyrins (FEP) is described in detail, the direct test being used for the analysis of blood specimens collected with anticoagulants and the spot test for analysis of blood specimens collected on filter paper. A method for determining the amount of blood absorbed by different lots of filter paper is described.

Rationale

The concentration of FEP (free erythrocyte porphyrins) in the blood increases exponentially in lead poisoning. The advantages of the FEP test are that this technique:

1. measures the FEP concentration in only 10 µl of blood
2. can be performed directly on blood collected by finger puncture (FEP direct test)
3. can be performed on a sample collected on filter paper (FEP spot test)
4. directly indicates metabolic effects of undue lead absorption
5. correlates clearly with blood Pb concentrations
6. is not subject to environmental contamination as is blood Pb.

Hemoglobin or hematocrit must be simultaneously measured if it is desired to determine the concentration of FEP in micrograms per gram of hemoglobin or in micrograms per deciliter of packed erythrocytes.

The FEP test is considered positive (1) (≥ three standard deviations above normal) when the FEP concentration is ≥60 µg/dl of blood or 5.3 µg/g of hemoglobin or ≥160 µg/dl of packed erythrocytes. All individuals that have a positive FEP test should have their blood lead concentration measured.

Iron deficiency anemia may also provoke a small to moderate increase in FEP. Such an increase may range up to 190 µg/dl of blood (or 17 µg/g of hemoglobin or 500 µg/dl of packed erythrocytes). Values above this range are observed exclusively in lead poisoning or in the rare genetic disorder, erythropoietic protoporphyria. Therefore, in individuals with moderately increased values, a differential diagnosis of iron deficiency anemia vs. lead poisoning must be obtained, by measuring the blood lead concentration.

The Direct FEP Test (2)

Principle

FEP (free erythrocyte porphyrins) are nearly completely extracted from the erythrocytes with ethyl acetate/acetic acid and transferred to 1.5 mol/liter HCl. Protoporphyrin IX is the major porphyrin extracted. The intense fluorescence of porphyrins in 1.5 mol/liter HCl is measured in a fluorometer and compared with that of a standard of protoporphyrin IX.

Celite is added to saline to facilitate transfer of the ethyl acetate extract, but it may be omitted. The extraction of FEP with this method is nearly complete (>96%) and the results are reproducible within ±2%.
The direct FEP test is performed on blood collected by finger- or venipuncture, with heparin or ethylenediaminetetraacetate (EDTA) as anticoagulants. The FEP content of the blood remains stable in the refrigerator for several months. (Heparinized blood may occasionally clot on storage: in this case measurements are reliable if the clots are removed, the hemoglobin is measured, and the results expressed in μg/g of hemoglobin. Despite this minor drawback, blood collected on heparin is preferable, as it can be used for measurements of blood Pb, Fe, and iron-binding capacity. Moreover, occasional lots of ethyl acetate may yield a lower extraction from EDTA than from heparinized samples.)

Blood collected by finger puncture directly on filter paper and shipped to a central laboratory for analysis is analyzed by the FEP spot test (see below).

Reagents

1. Celite in saline. Prepare a 50 g/liter suspension of Celite (No. C211; Fisher Scientific) in saline (9 g of NaCl/liter). This suspension keeps for at least two months.

2. Ethyl acetate/glacial acetic acid, 4/1 by volume. This solution keeps indefinitely.

3. Hydrochloric acid, 1.5 mol/liter. Dilute 125 ml of concentrated HCl to 1 liter with distilled water. This solution keeps indefinitely.

4. 1.5 mol/liter HCl saturated with ethyl acetate (for standards only). In a separatory funnel, mix 200 ml of 1.5 mol/liter HCl with 50 ml of ethyl acetate; agitate for 1 min. Let stand for 10 min until phases are separated. Remove the hydrochloric acid (lower) phase; store in a ground-glass-stoppered bottle. This solution keeps for at least three months.

5. Protoporphyrin IX standards. Preweighed vials containing exactly 5 μg of protoporphyrin IX (as the Zn complex) are commercially available (Porphyrin Products, P.O. Box 31, Logan, Utah 84321). Add 0.1 ml of the special solvent “Protosol” supplied with the vials; gently agitate, and let stand for 10 min. With a volumetric pipette, add 10 ml of reagent 4 and mix by inversion. This solution now contains 500 μg/liter. A working standard containing 50 μg/liter is prepared by precise 10-fold dilution with reagent 4. The standards are stable for 2–3 days, if not exposed to light (covered by aluminum foil). It is advisable to prepare a fresh standard daily for greater accuracy.

Instrumentation

Vortex-type mixer.

Test tubes, precleaned, disposable 13 × 100 mm (No. 14-958D; Fisher Scientific).

Filter fluorometer. We use an Aminco Microfluorometer (No. 4-7439) equipped with blue lamp (No. 4-7155), excitation filter with 405 nm peak (No. 4-7112), emission filter with 595 nm cut-off (No. 4-7117; Wratten 25), and round matched cuvettes (No. P183-501) and standard round cuvette holder. Any other type of fluorometer may be used if it has adequate sensitivity; it is necessary to obtain a measurable deflection of the needle with a concentration of 0.5 μg of protoporphyrin per liter of reagent 4; this is the concentration of the working standard diluted 100-fold.

Procedure

(a) Pipette 20 μl of blood to the bottom of a 13 × 100 mm test tube containing 0.3 ml of reagent 1. Test during the same day, or store in a refrigerator.

(b) Add to the tube containing the blood sample, 2 ml of the ethyl acetate/acidic acid mixture (reagent 2) and agitate for 10 s (vortex-type mixer).

(c) Centrifuge for 30 s at the medium speed of a table centrifuge (about 1200 rpm). Pour the supernate into another test tube. (The Celite/protein sediment adheres firmly to the bottom.)

(d) Add 2 ml of 1.5 mol/liter HCl (reagent 3) to the supernate and agitate the tube for 10 s (vortex-type mixer). A lower colorless HCl phase separates from an upper pink phase.

(e) Transfer the entire content of the tube (both phases) into a 10 × 75 mm round cuvette. Let stand for a few seconds to allow complete re-separation of the phases. Within 1 h, measure the fluorescence of the HCl phase directly in the cuvette. [This can be done with the Aminco microfluorometer; with other types of fluorometers it may be necessary to transfer only the HCl (lower) phase into the cuvette.] For samples with low FEP concentration (<100 μg/dl) the reading is obtained on the “0.1” multiplier scale of the Aminco microfluorometer. For samples with FEP concentration exceeding 100 μg/dl the reading is obtained on the “1” multiplier scale, and the value is multiplied by 10.

Calculations

The concentration of FEP, in μg/dl, is obtained by the formula:

\[
FEP = \text{fluorescence of sample} \times K \quad \text{(constant)}
\]

\[
K = \frac{C_s}{F_s} \times \frac{2.7}{0.02} \times 0.1
\]

where: \(C_s\) = concentration of protoporphyrin IX in the working standard (μg/liter); \(F_s\) = fluorescence of working standard (arbitrary units); 2.7 = final volume of HCl extract (increases from original 2 ml); 0.02 = volume of blood, in ml; 0.1 = to convert from liter to dl.

This formula can be used to compute FEP concentration when using any fluorometer. Omitting the 0.1, one obtains the results in SI units (μg/liter).

Direct Reading in μg/dl of Blood

With the Aminco microfluorometer (or another instrument of equal or better sensitivity) the concentration of FEP may be conveniently read directly in μg/dl of blood. This requires an appropriate adjustment of the sensitivity of this instrument. The reading is equal to μg/dl of blood when in the above formula \(K = 1\). With the working standard described above (50 μg of proto-
porphyrin IX per liter of reagent 4), the formula becomes:

\[ K = \frac{50 \, \mu g/liter}{F_s} \times \frac{2.7 \, ml}{0.02 \, ml} \times 0.1 = 1 \]

Thus: \[ F_s = \frac{(50)(2.7)(0.1)}{(1)(0.02)} = 13.5 \times 0.02 = 675 \]

Therefore, the sensitivity knob is adjusted to obtain with the working standard a reading of 67.5 on the multiplier scale of “1” (corresponding to 675 on the multiplier scale of “0.1”).

After the sensitivity has been adjusted for samples containing FEP concentrations below 100 \( \mu g/dl \) the reading will be obtained on the “0.1” multiplier scale directly in \( \mu g/dl \). For samples containing FEP concentration between 100 and 1000 \( \mu g/dl \) the readings will be obtained on the multiplier scale of “1” and the concentration of FEP in \( \mu g/dl \) is 10 times the value read.

Daily Standardization of the Microfluorometer for Direct Reading

(a) Blank the instrument with 1.5 mol/liter HCl.

(b) With the working standard of protoporphyrin IX (50 \( \mu g/liter \) of reagent 4), adjust the sensitivity knob to obtain a deflection of the needle to 67.5 on the “1.0” multiplier scale.

(c) With the multiplier set at “0.1,” the FEP concentration of unknown samples (\( \mu g/dl \)) is equal to the reading. If the reading is greater than full scale (100 \( \mu g/dl \)), turn the multiplier knob to “1.0” and multiply the reading obtained by 10.

Results

Normal values: \( \leq 59 \, \mu g/dl \) of blood.

Moderately elevated values (60–189 \( \mu g/dl \)) may be observed in lead intoxication and iron deficiency anemia.

Markedly elevated values (\( \geq 190 \, \mu g/dl \)) are observed in severe lead intoxication and in the rare genetic disorder erythropoietic protoporphyria.

The FEP Spot Test (3)

The FEP spot test gives results identical to the FEP test. Collection of the blood specimen on filter paper is convenient, requires no skill, the samples may be easily identified by writing on the paper, and may be transported or mailed without difficulties.

Sample

Blood is spotted directly from the finger onto the filter paper. Allow the paper to absorb the blood until a spot of approximately 1 cm\(^2\) is formed. Three spots are collected on the same piece of filter paper. (Only one spot is necessary for the test; however, an additional spot provides the opportunity for duplicate measurement and the third spot may be used for measurement of hemoglobin.)

The paper is allowed to dry in the air for at least 30 min. The blood spots on the paper should not touch anything while still wet. After the spots are dry, the papers may be stacked on each other.

The measurement of FEP may not be obtained until at least 4 h have elapsed from collection of the blood. (If a more urgent report is needed, it is then preferable to perform the regular FEP test.) The FEP remains stable on the paper for at least three months at room temperature. Exposure of the paper to prolonged direct sunlight should be avoided; refrigeration is not preferable.

Reagents

1. Celite in saline/Sterox. Prepare a 50 g/liter suspension of Celite (No. C211, Fisher Scientific) in saline (9 g of NaCl per liter). Add 0.5 ml of Sterox SE (Hartmann & Leddon, Philadelphia, Pa. 19143) per liter. This suspension keeps for at least two months.

2. Ethyl acetate/glacial acetic acid, 4/1 by volume. This solution keeps indefinitely.

3. Hydrochloric acid, 1.5 mol/liter. Dilute 125 ml of concentrated HCl to 1 liter with distilled water. This solution keeps indefinitely.

4. HCl, 1.5 mol/liter, saturated with ethyl acetate (for standards only). In a separatory funnel, mix 200 ml of 1.5 mol/liter HCl with 50 ml of ethyl acetate. Agitate for 1 min. Let stand for 10 min, until phases are separated. Remove the hydrochloric acid (lower) phase; store in a ground-glass-stoppered bottle. This solution keeps for at least three months.

5. Protoporphyrin IX standard. Preweighed vials containing exactly 5 \( \mu g \) of protoporphyrin IX (as the Zn complex) are commercially available (Porphyрин Products, P.O. Box 31, Logan, Utah 84321). Add 0.1 ml of the special solvent “Protosol” supplied with the vials; gently agitate, and let stand for 10 min. With a volumetric pipette add 10 ml of reagent 4 and mix by inversion. This solution now contains 500 \( \mu g/liter \). A working standard containing 50 \( \mu g/liter \) is prepared by precise 10-fold dilution with reagent 4. The standards are stable for two to three days, if not exposed to light (covered by aluminum foil). It is advisable to prepare a fresh standard daily for greater accuracy.

6. Cyanmethemoglobin reagent (modified Drabkin). To a 1-liter volumetric flask add 50 mg of potassium cyanide (KCN), 200 mg of potassium ferricyanide (K\(_3\)Fe(CN)\(_6\)), 140 mg of potassium phosphate, monobasic (KH\(_2\)PO\(_4\)), and 0.5 ml of Sterox SE (Hartmann & Leddon Co., Philadelphia, Pa. 19143). Dissolve in distilled water and dilute to 1 liter. The solution should be stored in a dark bottle.

7. Cyanmethemoglobin standard. Several certified standards are commercially available (e.g., from Hycel Inc., Houston, Tex. 77030).

Instrumentation

Office-type hand puncher, \( \frac{1}{4} \)-inch diameter.

Vortex-type mixer.

Table centrifuge.

Test tubes, precleaned, disposable, 13 \( \times \) 100 mm (No. 14-958D, Fisher Scientific).
Filter fluorometer. We use an Amino Microfluorometer (No. 4-7439) equipped with blue lamp (No. 4-7155), excitation filter with 405 nm peak (No. 4-7112), emission filter with 595 nm cut-off (No. 4-7117; Wratten 25), and round matched cuvettes (No. P-183-501) and standard round cuvette holder. Any other type of fluorometer may be used if it has adequate sensitivity; it is necessary to obtain a measurable deflection of the needle with a concentration of 0.5 μg of protoporphyrin per liter of reagent 4 (this is the concentration of working standard diluted 10-fold).

Filter paper, from Schleicher & Schuell, Keene, N.H. 03431; No. 903 (the type used in phenylketonuria screening of the newborn). The amount of blood absorbed by a ½-inch disk of paper should be known for each lot; if not, it must be determined (see below).

Clinical-type tube rotator.

Spectrophotometer. Any spectrophotometer or colorimeter of adequate sensitivity can be used, preferably an instrument that displays absorbance directly. We use the Model 300N (Gilford Instruments, Oberlin, Ohio 44074), which provides excellent sensitivity, permits reading of the supernate, without transfer, directly in grams of hemoglobin per 100 ml and can be automated to read up to 100 consecutive samples.

Procedure

(a) Punch from the blood-soaked area of the paper a ½-inch disk. Place the disk into a 13 × 100 mm test tube containing 0.3 ml of Reagent 1. Let stand at room temperature for at least 15 min.

(b) Add 2 ml of the ethyl acetate/acetic acid mixture (reagent 2). Agitate on a vortex-type mixer for 10 s.

(c) Centrifuge for 30 s in a table centrifuge at medium speed. Pour the supernate into another test tube. The Celite/protein and the filter paper remain adherent to the bottom of the first tube.

(d) Add 2 ml of 1.5 mol/liter HCl (reagent 3); agitate on a vortex-type mixer for 10 s; let stand for 30 s, until the phases are clearly separated.

(e) Transfer the entire contents of the tube (both phases) into a 10 × 75 cuvette. Let stand for 30 s or longer for complete re-separation of the phases.

(f) Within 1 h, measure the fluorescence directly in the cuvette. [This is possible with the Amino microfluorometer; with other types of fluorometers it may be necessary to transfer only the HCl (lower) phase, by using a capillary pipette and dropper, into the cuvette.] For samples with low FEP concentration, the fluorescence is determined on the “0.1” multiplier scale. For samples of higher concentration the reading is obtained on the “1” multiplier scale.

Calculations

The concentration of porphyrin in micrograms per deciliter of whole blood is obtained by the formula:

\[ \text{FEP} = \text{fluorescence of sample} \times K \text{ (constant)} \]

\[ K = \frac{C_s}{F_s} \times \frac{2.7}{v_b} \times 0.1 \]

where: \( C_s \) = the concentration of protoporphyrin IX working standard (in μg/liter); \( F_s \) = the fluorescence of working standard (in arbitrary units); 2.7 = the final volume of HCl extract (increases from original 2 ml); \( v_b \) = the volume of blood on the paper disk, in μl (may vary with lot of paper); 0.1 = factor to convert from liter to deciliter.

This formula can be used to compute FEP concentration in any fluorometer, once the value of \( v_b \) for the filter paper lot used has been determined, as described in a later section.

Direct Reading in Micrograms per Deciliter of Blood

With the Amino microfluorometer (or another instrument of equal or better sensitivity) the concentration of FEP may be conveniently read in micrograms per deciliter of blood by multiplying the reading times two. This requires an appropriate adjustment of the sensitivity of the instrument, taking into account also the volume of blood absorbed by the filter paper disk (\( v_b \)). The readings times two equals the micrograms per deciliter of blood when in the above formula \( K = 2 \). With the working standard described above (50 μg of protoporphyrin IX per liter of reagent 4), the formula becomes:

\[ K = \frac{50 \text{ μg/liter}}{F_s} \times \frac{2.7 \text{ ml}}{v_b \text{ ml}} \times 0.1 = 2 \]

Thus: \[ F_s = \frac{(50 \times 2.7)(0.01)}{(2)(v_b)} = \frac{13.5}{(v_b)} = \frac{6.75}{v_b} \]

Therefore the sensitivity knob is adjusted to obtain with the working standard a reading = 0.675/\( v_b \) on the multiplier scale of “1” (corresponding to 6.75/\( v_b \) on the multiplier scale of “0.1”). (Examples: \( v_b = 0.01 \); set sensitivity to read working standard = 67.5. \( v_b = 0.011 \); set sensitivity to read working standard = 61.5. \( v_b = 0.009 \); set sensitivity to read working standard = 75.0.) After the sensitivity is adjusted, for samples containing FEP concentrations of <200 μg/dl of blood, the readings will be obtained on the “0.1” multiplier scale and the concentration of FEP in micrograms per deciliter is equal to the reading times two. For samples containing FEP concentration above 200 μg/dl of blood, the readings will be obtained on the multiplier scale of “1” and the concentration of FEP in micrograms per deciliter of blood is determined by multiplying the reading times 20.

This standardization remains valid as long as filter papers from the same lot are used.

Daily Standardization of Microfluorometer for Spot Test

The fluorometer is calibrated, based on the above calculation, to give a reading for the working standard inversely proportional to the amount of blood absorbed by the filter paper disks from the lot used. Once this has been calculated, proceed as follows.
(a) Set the instrument on 1.5 mol/liter HCl.
(b) With the working protoporphyrin standard (50 μg/liter of reagent 4), adjust the sensitivity knob to obtain a deflection of the needle to the desired value (for the filter paper lot in use) on the “0.1” multiplier scale.
(c) With the multiplier set at “0.1” the FEP concentration of the unknown (in micrograms per deciliter of blood) is equal to the reading times two. If the reading is greater than full scale, turn the multiplier knob to “0.1” and multiply the reading obtained by 20.

Results of the FEP Spot Method

The results are the same as with the direct method, if expressed in micrograms per deciliter of blood. However, it is more precise to express the results in micrograms per gram of hemoglobin (Hb), dividing the FEP (μg/dl) by the hemoglobin value (g/dl) obtained from the same filter paper. The results in μg/g Hb (FEP/Hb ratio) are not influenced by anemia as are those in micrograms per deciliter (4).

Normal values: ≤59 μg/dl of blood; ≤5 μg/g Hb.
Moderately elevated values (60–189 g/dl; 5–17 μg/g Hb) may be observed in lead intoxication and iron deficiency anemia.
Markedly elevated values; ≥17 μg/g Hb) are observed in severe lead intoxication and in the rare genetic disorder erythropoietic protoporphyrinia.

Measurement of Hemoglobin on Filter Paper

Procedure

(a) Punch from the center of the blood-soaked area of paper a ¼-inch disk, letting it fall into a test tube (13 × 100 mm) containing 5 ml of cyanmethemoglobin reagent (reagent 6).
(b) Stopper with a rubber stopper and immediately mix in a clinical-type tube rotator for at least 15 min. Centrifuge for 15 min in a table-model centrifuge at maximum speed.
(c) With a transfer pipette, remove the supernate to another tube or directly to a cuvette. Take care to avoid disturbing the paper disk at the bottom. If paper debris is transferred, it may be necessary to centrifuge again.
(d) Measure the absorbance of the supernate at 540 nm vs. blank of reagent 6.
(e) Measure the absorbance of the standard reagent.
(f) Calculate the hemoglobin concentration in grams per deciliter by multiplying the absorbance of the sample times K (constant)

\[ K = \frac{hC_s}{A_s} \times \frac{5}{1000} \times \frac{1}{v_b} \]

where: \(hC_s\) = hemoglobin concentration of the standard (mg/dl); \(A_s\) = absorbance of the standard; 5 = volume of the diluent (ml); 1000 is to convert milligrams to grams; \(v_b\) = volume of blood on paper, in milliliters, which may vary with lot of paper.

This formula may be used with any instrument, once the value of \(v_b\) for the filter paper lot used has been determined as described in a later section.

Results of Hemoglobin Measurements

The values obtained between 7 and 15 g of hemoglobin per deciliter are identical to those obtained with the standard cyanmethemoglobin method. The reproducibility and variability are similar. For hemoglobin values below 7 g/dl, the filter paper method slightly underestimates the true value. For hemoglobin values exceeding 16 g/dl, the filter paper slightly overestimates the true value. These errors are only minor (less than 0.5 g/dl), and are due to the difference in viscosity of the blood at the extreme values, which changes the diffusion of blood in the filter paper.

The hemoglobin values remain unchanged on the filter paper for at least two months at room temperature. It is not necessary (or advisable) to refrigerate the paper. The elution of hemoglobin is not influenced by the humidity of the environment if the modified Drabkin solution described above is used.

Determination of Volume of Blood Absorbed by Filter Paper (\(v_b\)) in a Given Lot

Obtain a blood sample collected in heparin (Vacutainer green-top tube; Becton-Dickinson, Rutherford, N.J. 07070) with a hematocrit between 33–39%. (Normal adult blood has a hematocrit of 45%, which is too high. The hematocrit of normal blood may be adjusted to approximately 35% by adding 3 ml of AB plasma to 10 ml of blood and mixing). Keep the blood continuously agitated in a clinical rotator at room temperature.

With a capillary pipette, spot a drop (~30 μl) of blood on a small stopper covered with parafilm. Gently touching the drop of blood with the filter paper, allow the filter paper to absorb the blood until a spot of approximately 1 cm diameter is obtained. Be careful that the wet spot does not touch any surface. Repeat this procedure 20 times, to obtain 20 spots, replacing the tube with the blood on the running rotator in between every two or three spots. Allow the spots to dry overnight at room temperature in an atmosphere without extreme humidity.

From the same tube of blood, pipette exactly 20 μl with a Sahli hemoglobin pipette directly into 5 ml of cyanmethemoglobin reagent (reagent 6). Stopper the tube with a rubber stopper and invert three times. Repeat the procedure 20 times to obtain 20 samples. Be careful to replace the tube with the blood on the running rotator in between pipettings. Keep all stopped tubes overnight at room temperature, covered with aluminum foil.

The following day, prepare 20 tubes with 5 ml each of cyanmethemoglobin reagent (reagent 6). With an office puncher, punch a ¼-inch disk from the center of the blood spot on the filter paper directly into each test tube. Stopper the tubes and place on a clinical rotator for 15 min. Centrifuge for 15 min at the maximum speed of a table-model centrifuge. Transfer with a disposable
pipette the upper 3–4 ml of solution into another tube, being extremely careful not to disturb the sediment. Should any filter paper debris be left, centrifuge again and re-transfer.

Read the absorbance of the supernate in a spectrophotometer at 540 nm. Estimate the hemoglobin concentration (g/dl) of the samples by comparison with a cyanmethemoglobin standard.

Obtain the mean and standard deviation of the hemoglobin concentration in samples prepared with Sahli pipettes and from the paper disk eluates. The average volume of blood absorbed by the ¼-inch paper disk \( (v_b) \) is calculated from the following formula:

\[
v_b = \frac{x_f \times 0.02}{x_s}
\]

where: \( v_b \) = average volume of blood for ¼-inch disk of filter paper (µl); \( x_f \) = average Hb value of 20 filter paper disks; 0.02 = volume of blood in Sahli pipette (ml); \( x_s \) = average Hb value of 20 Sahli pipettes.

The value \( v_b \) is used to calculate both the FEP and the hemoglobin concentration on all samples collected on filter paper from the same lot number. Its value should be redetermined when another lot is used.

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References