Measurement of Free Erythrocyte Protoporphyrin in Blood Collected on Filter Paper as a Screening Test to Detect Lead Poisoning in Children

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ABSTRACT

A procedure for the measurement of free erythrocyte protoporphyrin (FEP) in a drop of blood collected on filter paper is described. The method is useful as a screening test for lead poisoning in children. Based on the FEP finding and blood lead tests, asymptomatic children are classified into four major categories. A course of action is suggested for each category.

Introduction

To cope with the large number of cases of lead poisoning in children, numerous screening tests have been proposed. At this time the screening tests considered most appropriate are based on the determination of erythrocyte protoporphyrin which is elevated in cases of undue lead absorption. One of the protoporphyrin tests, in which the blood is extracted with ethylacetate-acetic acid and hydrochloric acid, is called the free erythrocyte protoporphyrin test (FEP) and has been used with considerable success by the City of New York Department of Health Laboratories.

The free erythrocyte protoporphyrin (FEP) is elevated in cases of lead poisoning, iron deficiency anemia, and in the rare genetic disorder erythropoietic protoporphyria. In erythropoietic protoporphyria, uro- and coproporphyrins may also be elevated and could interfere with the determination of protoporphyrin IX. However, protoporphyrin IX may be distinguished from the other porphyrins by a differential solvent extraction and by their fluorescence at different wavelengths.

The FEP analysis can be performed more easily than a lead test. It is also less subject to extrinsic contamination, and can be performed on a specimen of blood collected from a finger prick. In addition, the FEP level is a better index of potential toxicity since it is less subject to
physiological fluctuation, and is usually elevated before clinical symptoms begin. Because of these advantages, one of the FEP tests was modified to make sample collection and processing easier.

Since the test is not specific for lead intoxication, a specimen should be collected for a micro blood lead determination at the same time the FEP specimen is collected. The micro blood lead test should be performed on all specimens whose FEP level is found to be elevated. Because micro leads are frequently elevated owing to contamination, venous blood levels are recommended for confirmatory purposes. When the FEP is normal, no further action is indicated.

Materials and Methods

**Principle**

1. Disks punched from the same lot of filter paper which have been previously saturated with blood contain equal amounts of blood.

2. Standard disks can be prepared from blood specimens of known FEP concentration.

3. The fluorescence of the disks obtained from a patient’s blood is compared to that of the standard disks. From this data, the FEP value for the patient’s blood can be calculated.

4. The concentration of FEP is exponentially related to the blood lead level.

**Reagents**

All reagents were used without further purification. Reagents include filter paper 8.2 cm in diameter* and zinc protoporphyrin IX.†

**Standard Solution**

A vial containing a 5.6 µg of zinc protoporphyrin IX is used. One hundred µl of a special solvating solution, Protosolv†, are added to the vial which is shaken for ten min. Then 10.0 ml of 1.5 N hydrochloric acid, which has been previously saturated with ethylacetate-acetic acid (4:1 by volume), are added. On the addition of the hydrochloric acid, the zinc protoporphyrin IX forms 5.0 µg of protoporphyrin IX. This stock solution then contains 0.50 µg per ml protoporphyrin IX. The protoporphyrin is diluted tenfold with the same hydrochloric acid to form a working solution containing 0.05 µg per ml which is used each day to calibrate the spectrofluorometer. The maxima wavelengths for the protoporphyrin IX are an excitation wavelength of 405 nm and an emission wavelength of 610 nm. These solutions must be freshly prepared each day. The hydrochloric acid is saturated with the ethylacetate-acetic acid solution so that the protoporphyrin IX is dissolved in the same solvent as the unknown blood specimens.

**Special Apparatus**

All fluorescence measurements were made with a spectrofluorometer‡ employing a red sensitive R 136 photomultiplier tube. The spectrofluorometer must have sufficient sensitivity so that 0.05 µg per ml protoporphyrin IX in 1.5 N HCl (without prior ethylacetate-acetic acid saturation) can be set to read 0.80 relative intensity at the protoporphyrin IX maxima (excitation 405 nm and emission 610 nm) wavelengths.

**Procedure**

The patient’s finger is scrubbed for one minute with a gauze pad which has been soaked in a solution containing 2 percent citric acid and 70 percent ethanol. After drying, a film of flexible collodion is then applied. When this dries the finger is punctured and four blood spots are collected on the filter paper. The blood spots should be about ½ inch in diameter. When the blood dries, a disk is punched

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* No. 903, Schleicher and Schuell, Keene, NH.
† Porphyrin Products, Logan, UT.
‡ American Instrument Company SPF125.
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from the blood soaked area of the filter paper. The remaining three spots are retained for possible use in the micro lead test. The ¼ inch blood-disk is placed into a test tube and 0.3 ml of an aqueous solution which contains 5 percent celite and 0.5 percent sterox is added. The disk is allowed to stand at room temperature for 15 min with occasional shaking. Two ml of a solution of ethylacetate-acetic acid (4:1) are added. The contents of the tube are thoroughly mixed on a Vortex mixer for ten sec. The test tube is then centrifuged at 1800 rpm for 30 sec. The supernatant is poured into another test tube. Two ml of 1.5 N hydrochloric acid are added and both layers are agitated on a Vortex mixer for ten sec. After the two phases have separated, the upper ethylacetate layer is removed by aspiration, and the hydrochloric acid solution is transferred to a spectrofluorometer cuvet. The relative intensity of the hydrochloric acid is read in a spectrofluorometer at an excitation wavelength of 405 nm and an emission wavelength of 610 nm which has been calibrated with 0.05 μg per ml solution of protoporphyrin IX. A reagent blank, consisting of a disk of filter paper without blood, is analyzed simultaneously. The relative intensity obtained for the blank is subtracted from each test specimen.

Preparation of Standards

Five venous blood specimens which have been previously analyzed for lead are used to prepare an FEP standard curve. Three of the selected specimens should have lead levels between 40 to 60 μg per 100 ml blood, and one specimen should have a lead level above 60 μg per 100 ml blood. The FEP levels of these blood specimens are determined using the procedure described previously except that 20 μl of whole blood are used instead of the blood soaked disk and 0.10 ml of the mixture containing celite and sterox.

The FEP concentration is calculated in μg per 100 ml whole blood by multiplying the fluorescence of the blood by a constant factor.

where:

\[ K = \frac{Cs \times 2.5 \times 100}{Fs \times 0.020} \]

Cs = concentration of protoporphyrin IX standard (0.05 μg per ml),
Fs = fluorescence of the protoporphyrin IX standard,
0.020 = volume of blood used, and
2.5 = increase in volume of the 2 ml hydrochloric acid as result of mixing with the ethylacetate-acetic acid solution.

If the spectrofluorometer is set at a relative intensity of 0.625 with the protoporphyrin IX standard, then \( K = 1000 \).

Approximately 12 portions in the amount of 20 μl each of the various blood standards of known FEP values are spotted one cm from the circumference of the filter paper. Enough standard blood disks should be prepared to last one month. After the blood spots have dried, the filter paper is placed in a Petri dish and stored in a refrigerator. Blood disks prepared in this manner will give the same slope (in our laboratory 0.33 ± 0.03) for a period of one month.

The mean values and standard deviations for three standards analyzed daily for a period of two weeks were 33 ± 5, 79 ± 8 and 196 ± 11.

Discussion

A simple micro-method is described for the detection of lead poisoning in children using blood collected on a piece of filter paper. It correlates well with the liquid procedure utilizing 20 μl of whole blood. It should be pointed out that the elaborate finger cleaning described under Procedure is designed to eliminate contamination for the micro lead deter-
mination and is not actually required for the FEP test.

Sources of Error

Certain lots of ethylacetate were found to contain an impurity which quenched the FEP fluorescence. The suitability of the ethylacetate can be determined in the same manner as described for checking the spectrofluorometer. In this case, however, the hydrochloric acid is saturated with the ethylacetate-acetic acid solution. The instrument should not read significantly lower than the same solution without ethylacetate-acetic acid saturation.

Different lots of filter paper may absorb different volumes of blood. Therefore, the distribution of the filter paper should be controlled by the laboratory. New lots of punched filter paper should be tested before distribution to insure that the standards are prepared on paper with similar absorption characteristics as that used for the collection of the specimens.

Normal Range for Children

An FEP level less than 60 μg per 100 ml whole blood is considered normal and no further testing is required. An FEP level of 60 μg per 100 ml whole blood or higher is considered positive and a micro lead determination should be made on the remaining blood disks, employing the Delves cup atomic absorption procedure.

Table I

<table>
<thead>
<tr>
<th>Free Erythrocyte Protoporphyrin μg per 100 ml</th>
<th>Blood Lead Level μg per 100 ml</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Micro</td>
</tr>
<tr>
<td>&lt;39</td>
<td>≤60</td>
</tr>
<tr>
<td>60-109</td>
<td>Ia</td>
</tr>
<tr>
<td>110-189</td>
<td>Ia</td>
</tr>
<tr>
<td>&gt;190</td>
<td>EPP</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Class</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Normal - no immediate follow-up indicated.</td>
</tr>
<tr>
<td>Ia</td>
<td>Repeat screening test within 30 days. If free erythrocyte protoporphyrin and micro lead fall into Class II a second time, recall for venous blood lead within 30 days.</td>
</tr>
<tr>
<td>II</td>
<td>Refer to treatment agency.</td>
</tr>
<tr>
<td>IIa</td>
<td>Refer to treatment agency.</td>
</tr>
<tr>
<td>IIb</td>
<td>Refer to treatment agency.</td>
</tr>
<tr>
<td>III</td>
<td>Collect blood for venous lead determination and refer child to treatment agency. Do not wait for venous lead result.</td>
</tr>
<tr>
<td>IV</td>
<td>Evaluate for erythropoietic protoporphyria.</td>
</tr>
</tbody>
</table>

Resumé of Clinical Interpretations for Children

Children may be classified into four major categories depending on the results of the FEP and blood lead tests. These categories, as shown in Table I, reflect the degree of risk to the asymptomatic child and suggest a course of action which has been adopted by New York City Child Health Stations and Pediatric Treatment Clinics. These recommendations are based on statements made by the U. S. Department of Health, Education and Welfare, Center for Disease Control in March 1975.10

A certain degree of flexibility is permitted in the scheduling and type of specimens collected in follow-up testing. For example, Class III children between the ages of one and four years, with FEP values between 150 and 189 and living in suspect housing, need to be followed more closely, especially in the summer months. Less frequent follow-ups are required for children five years of age with FEP values between 110 and 150 who are now living in housing free of lead paint. In the former class, follow-up within 30 days is required while in the latter case, a micro blood lead and FEP test conducted at 6 to 12 week intervals would be considered appropriate.

As in other cases, some degree of judgment must be exercised. No child is referred to a treatment agency on the basis of a FEP or micro lead test alone unless the child is in Class IV or has symptoms.
which may be attributed to lead poisoning. At every opportunity, parents are cautioned as to the danger of lead poisoning from the ingestion of paint chips and the need for periodic testing of children who live in deteriorated housing.

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References