Empirically Determined Lead-Poisoning Screening Cutoff for the Protofluor-Z Hematofluorometer

Noel V. Stanton,¹ Elaine W. Gunter,² Patrick J. Persons,³,⁴ and Patricia H. Field¹

A recently introduced hematofluorometer, the "Protofluor-Z" (Helena Laboratories, Beaumont, TX), has several novel features, most notably reporting units expressed as the molar ratio of zinc protoporphyrin (ZPP) to heme, i.e., micromoles of ZPP per mole of heme. We analyzed human blood specimens on the Protofluor-Z and by an ethyl acetate/acetic acid extraction procedure. Data from three laboratories were pooled and used to provide a comparison of the two methods. Results indicate that, with the Protofluor-Z, a value of 70 µmol of ZPP per mole of heme is approximately equivalent to the recommended screening cutoff of 35 µg of erythrocyte protoporphyrin per 100 mL of whole blood used in programs for pediatric lead-poisoning prevention. This empirically determined value is slightly lower than either that recommended by the manufacturer or a theoretical cutoff value that was determined mathematically.

Additional Keyphrases: zinc protoporphyrin · heme

Measurement of erythrocyte protoporphyrin (EP), currently the preferred screening test for detection of pediatric lead poisoning, is also a sensitive indicator of iron deficiency anemia (1, 2).¹ Hematofluorometers (HF) are used extensively to obtain these screening measurements, because they provide immediate results and are relatively simple and inexpensive to operate.

The hematofluorometer is essentially a small, dedicated, filter fluorometer in which the principle of front-surface fluorometry is utilized to obtain results from a thin smear of whole blood placed on a coverslip and inserted into the instrument (3). The intensity of the analytical signal produced by the instrument is a function of the molar ratio of fluorescing zinc protoporphyrin (ZPP) to light-absorbing heme. ZPP, the predominant species of protoporphyrin present in human erythrocytes (4), generally constitutes approximately 90% of the total (5). The molar ratio of ZPP to heme is independent of the sample hemoglobin concentration and the volume of blood measured. In most HF instruments, however, the final result is expressed as micrograms equivalent EP per 100 mL of whole blood, to convey correlation with extraction methods. To allow expression of results in these units, a representative hemoglobin concentration (113 g/L) or corresponding hematocrit (35%) is assumed for all pediatric specimens.

The ZPP/heme ratio has been acknowledged to be a more reliable indicator of protoporphyrinemia (6, 7), but few data are available defining the clinical significance of measurements expressed in these terms. Much of the clinical data on EP, and the database for the Centers for Disease Control's risk classifications for pediatric subclinical lead intoxication (1), were obtained by using extraction with ethyl acetate/acetic acid followed by conventional fluorometric measurement, and reported as micrograms of EP per 100 mL of whole blood (8-10), the same concentration units used for reporting values for blood lead. The calibration of conventional hematofluorometers, such as the ESA and Aviv brands, can ultimately be traced to an extraction method. Therefore, hematofluorometry and extraction are expected to produce comparable results.

A recently introduced hematofluorometer, the "Protofluor-Z" (Helena Laboratories, Beaumont, TX 77704) has several features that differentiate it from other such instruments. Most notably, it reports results as the molar ratio: micromoles of ZPP per mole of heme. In addition, a derivatizing reagent unique to this instrument is used to eliminate the need for manual oxygenation of specimens (3, 11).

Few independent data are available for comparing results from the Protofluor-Z with those obtained by extraction methods and, in turn, with extraction-based action values (i.e., cutoff values) for pediatric screening. Of particular importance is the determination of a ZPP value in micromoles per mole of heme that is equivalent to 35 µg of EP per 100 mL of whole blood, the recommended screening cutoff for detection of pediatric lead intoxication and (or) iron deficiency (1, 2, 12).

In this study, we have compared Protofluor-Z results empirically with extraction values, and propose an appropriate screening cutoff value for the Protofluor-Z. A mathematical formula to interconvert the two reporting units is also presented, and its relative merits are discussed.

Materials and Methods

Participating laboratories. Data for this study were generated in the Toxicology Section of the Wisconsin State Laboratory of Hygiene, the Laboratory of Inorganic and Nuclear Chemistry at the New York State Department of Health, and the Nutritional Biochemistry Branch of the Centers for Disease Control. Each of the participating laboratories performs a large number of routine EP determinations and participates as a reference laboratory in state and federal proficiency-testing programs, giving each participant extensive experience with the accurate measurement of EP.

Extraction procedure. The extraction procedure used in the study is similar to previously published methods (8, 9),

---

¹Toxicology Section, Wisconsin State Laboratory of Hygiene, 465 Henry Mall, Madison, WI 53706.
²Nutritional Biochemistry Branch, Centers for Disease Control, Atlanta, GA 30333.
³Laboratory of Inorganic and Nuclear Chemistry, Wadsworth Center for Laboratories and Research, P.O. Box 509, New York State Department of Health, Albany, NY 12201.
⁴Department of Environmental Health and Toxicology, School of Public Health Sciences S.U.N.Y., Albany, NY 12237.
⁵Nonstandard abbreviations: EP, erythrocyte protoporphyrin; HF, hematofluorometer; ZPP, zinc protoporphyrin; HCT, hematocrit; Hgb, hemoglobin; and PPIX, protoporphyrin IX.
⁶The use of trade names or commercial materials associated with this study does not confer an endorsement by the U.S. Public Health Service, the Centers for Disease Control, the New York State Department of Health, or the Wisconsin State Laboratory of Hygiene.
⁷Received May 26, 1989; accepted July 5, 1989.
but with some modifications that were agreed upon by the authors and several other laboratories that routinely determine EP. Briefly, one part of well-mixed whole blood is diluted with four parts of water. A 50-µL aliquot is transferred to a culture tube and mixed with 1.0 mL of a 4/1 (by vol) ethyl acetate/acetic acid mixture. This mixture is centrifuged for 3 min at approximately 1500 × g, and the supernate is then mixed with 1.0 mL of 15.5 mol/L HCl solution. Protoporphyrin IX in the aqueous layer is then measured with a spectrophotometer at excitation and emission wavelengths of 408 and 662 nm, respectively. A red-sensitive photomultiplier tube is used to improve sensitivity. De-ionized water is used throughout, and all chemicals are reagent grade. (Detailed copies of the procedure are available from the authors on request.)

Hematofluorometer procedure. The Protofluor-Z hematoflurometers were operated according to the manufacturer's instructions. Before each use we calibrated the instruments by using two calibrator solutions provided by the manufacturer. For specimen analysis, we added about 25 µL of well-mixed blood to about 50 µL of "Protofluor" reagent in a glass culture tube. The approximate 1:2 volume ratio of sample to reagent is specified by the manufacturer as one drop of blood and two drops of reagent (13). We noted during the study that changes in this ratio altered the results, with a higher proportion of reagent causing an increase in results (data not shown). Throughout the study we used the ratio specified by the manufacturer. The blood and reagent were mixed and poured onto a disposable no. 1 grade glass coverslip (Corning Glass Works, Corning, NY 14831), which we then inserted into the instrument. The result is given as a digital readout. We rechecked instrument calibration after each set of measurements.

Blood specimens. Blood specimens for the study were collected by hospitals and local public health agencies and submitted to the participant laboratories for routine determination of EP and (or) blood lead. All specimens were collected by venipuncture and anticoagulated with EDTA or heparin. The sample population included both children and adults. Specimens were kept refrigerated at 4 °C and protected from light before analysis. Hematofluorometer measurements were obtained within three days of specimen receipt. Hemolysed and visibly decomposed specimens were rejected. Extraction measurements were obtained within one week of specimen receipt.

Hematocrits (HCT) were measured with the use of a microhematocrit centrifuge and glass capillary tubes, or by measuring hemoglobin (Hgb) concentration with an IL 282 Co-Oximeter (Instrumentation Laboratory Inc., Lexington, MA 02173) calibrated to a standard cyanmethemoglobin method (14), and converted to equivalent hematocrit with the following formula: measured Hgb (g/L) × 0.31 = HCT, where 0.31 = average ratio of Hgb (g/L) to HCT (15).

Results

We analyzed 159 blood specimens for EP by extraction, and for ZPP/heme by using the Protofluor-Z. Extraction results were corrected for hematocrit to yield results in micrograms of EP per 100 mL of erythrocytes and plotted vs the Protofluor-Z results. Simple linear-regression analysis of the data produced a best-fit straight line. Outliers were identified by inspecting the standardized residuals and rejecting data points that were more than two standard deviations from the line (five data points were rejected). The corrected line was then replotted and is shown in Figure 1. Results from the two methods show excellent correlation and linearity (r² = 0.963) over the range of values observed. Extraction results ranged from 27 to 386 µg of EP per 100 mL of erythrocytes (10 to 153 µg per 100 mL of whole blood), encompassing the range of results commonly encountered in pediatric screening for lead intoxication and iron deficiency.

A value of 100 µg of EP per 100 mL of erythrocytes by the extraction method was calculated as corresponding to the recommended pediatric screening cutoff of 35 µg of EP per 100 mL of whole blood with an assumed HCT of 35%. Using this value in the equation of the regression line from Figure 1 (y = 0.583x + 11.9) results in a predicted equivalent screening cutoff value of 70 µmol of ZPP per mole of heme on the Protofluor-Z. This value differs slightly from that provided by the manufacturer, 75 µmol of ZPP per mole of heme. The 95% confidence interval of the slope indicates a variance of 68–72 µmol of ZPP per mole of heme, assuming that statistical variance lies only in the ZPP/heme data.

We investigated the possibility that a mathematical conversion might also be used to estimate a screening cutoff in terms of ZPP/heme that would be equivalent to 35 µg of EP per 100 mL of whole blood. We used the following derivation to determine an approximate conversion factor.

Assuming that ZPP (by HF) ≈ EP (by extraction), on a weight basis, then:

\[
\frac{\mu\text{g of EP}}{100 \text{ mL of whole blood}} = \frac{\mu\text{g of ZPP}}{100 \text{ mL of whole blood}}
\]

6 On a molar basis, approximately 90% of the total EP present in humans exists as ZPP (6). Most of the remaining protoporphyrin exists as the (zinc) free protoporphyrin IX (PPIX). Extraction methods measure essentially all the EP present, but the acidic solutions used in the process dissociate the zinc from ZPP, leaving all the measured protoporphyrin in the PPIX form. Hematoflurometers preferentially measure only the ZPP fraction. Therefore, on a weight basis: EP (by extraction) × 0.9 × 625.6/562.3 = ZPP (by HF) where 0.9 = molar ratio ZPP/EP, 625.6 = molecular mass of ZPP, and 562.3 = molecular mass of PPIX. Upon multiplication, this resolves to EP × 1.0 ≈ ZPP, so EP ≈ ZPP.

Fig. 1. Simple linear-regression analysis of Protofluor-Z vs extraction EP results after deletion of outliers. The outer line define the 95% confidence limits. O, outliers

CLINICAL CHEMISTRY, Vol. 35, No. 10, 1989 2105
The absolute accuracy of the Protofluor-Z was not addressed in this study. The accuracy of calibrator solutions supplied by the manufacturer, a mixture of ZPP and hemin, has yet to be independently determined. Though not designed for use with other instruments, the Protofluor calibrator solutions were evaluated in another study and found to be unsuitable for use with ESA and Aviv hematofluorometers (18). The Protofluor derivatizing reagent used with clinical blood specimens also complicates any assessment of the accuracy of the Protofluor-Z. This reagent, essentially a dilute aqueous solution of potassium cyanide, converts other hemoglobin species to cyanohemoglobin; its use reportedly removes a major source of error associated with hematofluorometric measurements (19). Further investigation is needed to ascertain the absolute accuracy of the Protofluor-Z, and to define more clearly the relationship of the ZPP/heme ratio to relative risk in pediatric lead screening.

This work was supported in part by grants MCJ 555103 (SLH) and MCJ 365667010 (NYS) from the U.S. Department of Health and Human Services, Bureau of Maternal and Child Health and Resources Development, Rockville, MD. We also acknowledge Helena Laboratories for providing supplies used in the study. We are grateful to Dr. Robert Labbé and Ms. Rebecca Bettmer for their helpful discussions on this work. We acknowledge the numerous laboratory staff who assisted in taking measurements used in the study.

References

Incorporating the measurement of the sample’s Hgb (g/100 mL of blood) into the equation yields:

\[
\frac{\mu g \text{ of EP}}{g \text{ of Hgb}} \times (1.598 \times 10^{-3}) = \frac{\mu mol \text{ of ZPP}}{g \text{ of Hgb}}
\]

\[
\frac{\mu g \text{ of EP}}{g \text{ of Hgb}} \times (1.598 \times 10^{-3}) = \frac{64,458 \text{ g of Hgb}}{mol \text{ of Hgb}}
\]

\[
\frac{\mu mol \text{ of ZPP}}{mol \text{ of Hgb}} = \frac{\mu g \text{ of EP}}{g \text{ of Hgb}} \times 103.0
\]

\[
\frac{\mu g \text{ of EP}}{g \text{ of Hgb}} \times 103.0 \times \frac{1}{4 \text{ mol of heme}} = \frac{\mu mol \text{ of ZPP}}{mol \text{ of heme}}
\]

Assuming \( g \text{ of Hgb} \times 3.1 = HCT (\%) \), then:

\[
\frac{\mu g \text{ of EP}}{\text{sample HCT (\%)} \times 80} = \frac{\mu mol \text{ of ZPP}}{\text{mol of heme}}
\]
We compared measurements of daily urine oxalate excretion in urines collected at the prevailing urine pH with measurements of urine oxalate excretion in urines collected in 20 mL of 6 mol/L HCl. We studied eight healthy adults fed constant diets. Urines were collected during control conditions and, in each subject, during the administration of NaCl, KCl, NaHCO₃, or KHCO₃, 90 mmol/day. Daily urine oxalate excretion calculated for collections made in acid averaged 271 (SD 79) μmol/day and did not vary with any of the salt supplements. When urines were collected at ambient urine pH (average 5.94, SD 0.23) during control conditions, and during the administration of NaCl or KCl, urine oxalate excretion averaged 263 (SD 88) μmol/day, a value not different from that for collections in acid. However, when urine was collected with no pH adjustment during NaHCO₃ or KHCO₃ administration (average pH 6.90, SD 0.14), apparent urine oxalate excretion averaged 398 (SD 132) μmol/day, significantly (P < 0.025) exceeding the mean observed when urines were collected in acid. Moreover, the percentage increase in apparent oxalate excretion increased with urinary pH. These observations reinforce recommendations that urine specimens for measurement of oxalate be collected in acid to avoid the increase in apparent oxalate content that occurs during collection of alkaline urines. This increase presumably results from the well-known in vitro nonenzymatic conversion of ascorbate to oxalate.

Additional Keyphrases: calculous disease · variation, source of

Currently, ~80% of the kidney stones examined in the United States are composed in part of calcium oxalate (J).