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STANDARDIZATION OF METHODOLOGY FOR ZINC PROTOPORPHYRIN IN ERYTHROCYTES: INCLUDING NEW SPECTRAL DATA ON ZINC PROTOPORPHYRIN

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ABSTRACT

Erythrocyte protoporphyrin tests are widely used in epidemiologic studies on lead exposure and their use in men occupationally exposed to lead has been proposed. In lead poisoning and iron deficiency, the circulating erythrocytes contain zinc protoporphyrin (ZnPROTO).

The subject of this paper are studies on the determination of ε_{mmol} of zinc protoporphyrin, as well as the development of an acetic acid-acetone extraction system in which mixtures of ZnPROTO and free PROTO in blood can be detected and ZnPROTO measured quantitatively.

We report here that circulating erythrocytes may contain mixtures of ZnPROTO and free PROTO in other conditions, including sickle cell disease and undernutrition. The clinical data indicate that free PROTO is not related to lead absorption. The new spectral data for ZnPROTO should facilitate further the standardization of various micro methods for its determination.

Erythrocyte protoporphyrin tests are widely used in epidemiological studies on lead exposure. Recent studies suggest that they may also be useful in the biological monitoring of lead workers. In lead poisoning and latent iron deficiency, the circulating erythrocytes contain zinc protoporphyrin[6] which can be measured either directly[7] or indirectly. For example, in the classical ethyl acetate-acetic acid-hydrochloric acid extraction procedures for "FEP", zinc is removed from the porphyrin nucleus, so that zinc protoporphyrin in circulating erythrocytes is actually measured as "free". When zinc protoporphyrin is the only species of porphyrin present in erythrocytes, zinc protoporphyrin and FEP methods should, in theory give equivalent results. However, ratios varying between 0.56 and 1.38 have been reported[3,5], and the differences between methods have been attributed to differences in extraction efficiency. While this may be true in some instances[3], it is also possible that the millimolar absorptivity (ε_{mmol}) values used to calculate the concentrations of zinc protoporphyrin and protoporphyrin IX in standard solutions may not be reliable. If so, systemic errors would result. To examine this possibility, we have determined the

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millimolar absorptivity of zinc protoporphyrin on a carefully characterized reference sample of this substance and developed a quantitative acetic acid--acetone extraction method which is specific for zinc protoporphyrin in erythrocytes.

MATERIAL AND METHODS

Several samples of zinc protoporphyrin reference material were obtained from different sources. Thin layer chromatography showed that most contained a brown pigment, while a few contained traces of protoporphyrin IX. The contaminated samples were discarded. A sample without these impurities was studied further. The method of preparation of this sample indicated that dimethylformamide, ammonia and acetate might be present in the final product. (Zinc protoporphyrin, Lot 130, Porphyrin Products, P. O. Box 31, Logan, Utah, U.S.A. 84321). This lot was prepared as follows: Protoporphyrin IX and zinc acetate were dissolved in DMF in a molar ratio of 1 to 1.1 respectively. The solution was heated and monitored spectrophotometrically until 100% incorporation of Zn into PROTO was achieved. The sample was cooled, extracted with ethyl acetate and washed with water repeatedly. Zn PROTO was then extracted from ethyl acetate into dilute NH_4OH and precipitated by lowering the pH to the isoelectric point with acetic acid. Nuclear magnetic resonance spectroscopy revealed not only that the zinc protoporphyrin molecule was intact, but also that dimethylformamide was present as a ligand. Mass spectroscopy indicated the absence of ammonia, while infrared spectroscopy suggested the possibility that traces of acetate might be present. Elemental analysis showed that the amount of zinc protoporphyrin in the sample, as calculated from its zinc content, was 84%. Millimolar absorptivity was then determined on weighed portions of the same sample after correction for its actual zinc protoporphyrin content. The results are shown in Table 1. The millimolar absorptivity of zinc protoporphyrin in pyridine is 221 and in the acetic acid-

TABLE 1
Spectral reference data zinc protoporphyrin IX.

SOLVENT	SORET MAX. nm	MILLIMOLAR ABSORPTIVITY (Σ mmol)
Pyridine	427	221
Acetone containing 8.2% Acetic Acid	417	211
PROTOPORPHYRIN IX (FREE BASE)		
1.0 mol HCl/liter	408	241*

*From Caughey, W.S., Adler, A., Burnham, B. F., et al. in "Specifications and Criteria for Biochemical Compounds," p. 185, National Academy of Sciences, Washington, D.C., U.S.A., 1972.

-acetone solvent it is 211. The value shown in Table 1 is substantially higher than previously reported values of 126 and 160 for its absorptivity in pyridine. In the earlier reports, extensive characterization of the reference sample of zinc protoporphyrin used was not reported. The millimolar absorptivity for "free" protoporphyrin IX in 1 molar hydrochloric acid shown in Table 1 is the value recommended by the National Academy of Sciences, Washington, D. C. We were able to confirm this value within 3%.

Next we developed a quantitative micro fluorometric method for zinc protoporphyrin in erythrocytes. Briefly, the method, which is described more fully elsewhere[2] is as follows: pipette 10 μl of EDTA-anticoagulated whole blood into 125 μl of 0.5 molar acetic acid, jiggle to mix and hemolyze the blood and *immediately* add 1.5 ml of acetone. After mixing and centrifugation, zinc protoporphyrin is measured fluorometrically. Standards are prepared freshly each day from chromatographically-pure protoporphyrin IX free base (Porphyrin Products, P. O. Box 31, Logan, Utah, U.S.A. 84321) which is mixed with zinc acetate in dimethylformamide and reacted at 75 °C. Contamination with pigments is avoided by this technique. Conversion of protoporphyrin IX to zinc protoporphyrin is complete within two to three minutes. The reaction is monitored spectrophotofluorometrically.

RESULTS AND DISCUSSION

Figure 1 shows fluorescence spectra for zinc protoporphyrin and free protoporphyrin IX, as well as for a mixture of the two in acetic acid-acetone solvent. Panel A on the left shows fluorescence emission spectra for zinc protoporphyrin and free protoporphyrin, separately. Panel B on the right shows the emission spectra observed when a mixture of zinc protoporphyrin and free protoporphyrin is present. Thus, the conversion of free protoporphyrin to zinc protoporphyrin during the preparation of standards is readily monitored. With the use of a narrow band pass secondary interference filter centered at 597 nm, zinc protoporphyrin can be measured quantitatively with a filter fluorometer without interference due to free protoporphyrin.

Next, 58 samples were split for analysis by both a standard micro FEP method[1] and the acetone-acetic acid method for zinc protoporphyrin. These samples were selected for comparison after spectrophotofluorometry confirmed the absence of free protoporphyrin. The results, as shown in Figure 2, are expressed as micromoles zinc protoporphyrin or free protoporphyrin/liter of erythrocytes. Linear regression analysis indicates close correlation between the zinc protoporphyrin and FEP methods when only zinc protoporphyrin is present ($r = 0.994$), a negligible intercept (−0.161) and nearly equivalent results (slope = 1.042). The standard method for FEP calls for 20 μl of blood. When the volume of blood is reduced to 10 μl in the FEP method, a slope of 0.986 is obtained. These and other studies provide independent lines of evidence that the millimolar absorptivity of zinc protoporphyrin IX in this particular acetic acid--acetone solvent system is 211.

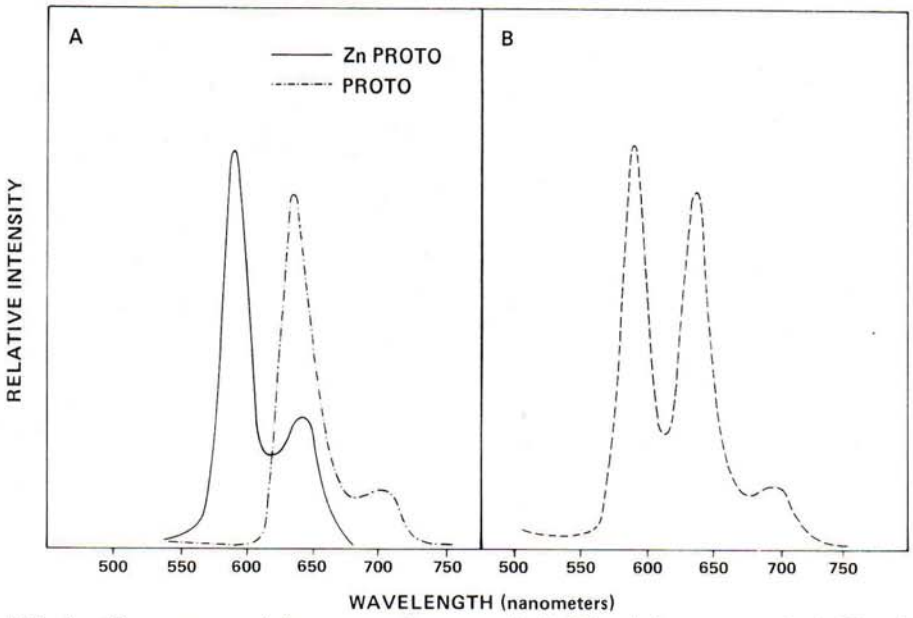

FIG. 1 – Fluorescence emission spectra of protoporphyrin IX and zinc protoporphyrin IX and mixtures of both in acetic acid-acetone solvent.

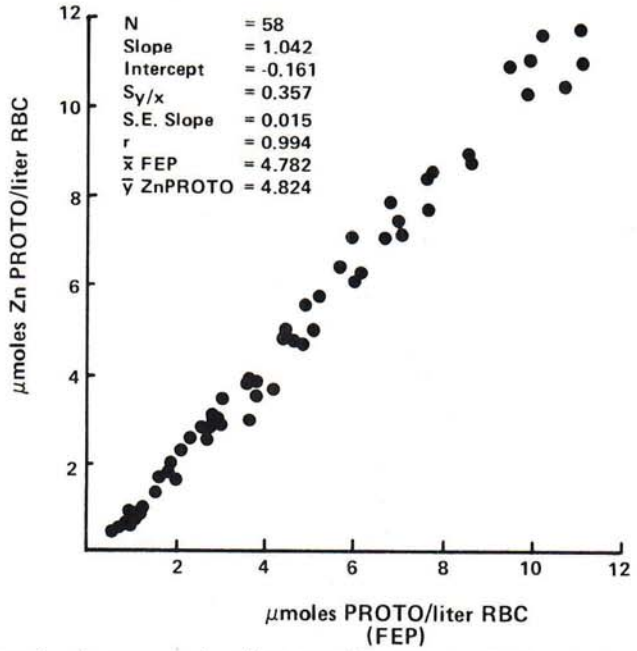

FIG. 2 – Comparison between acetic acid-acetone (zinc protoporphyrin) and ethyl acetate/acetic acid/HCl (FEP) methods in 58 blood samples containing zinc protoporphyrin only.

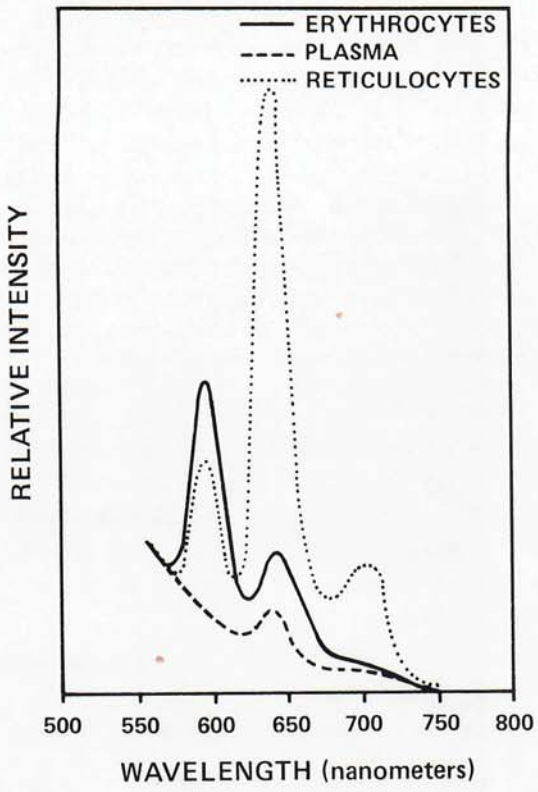

FIG. 3 – Distribution of zinc protoporphyrin IX and protoporphyrin IX (free base) in mature erythrocyte and reticulocyte-rich fractions of human cord blood as detected by fluorescence emission spectra.

During the course of this work, spectrophotofluorometry has revealed mixtures of zinc protoporphyrin and free protoporphyrin IX in many clinical samples. When both FEP for total porphyrin and zinc protoporphyrin are measured quantitatively, the amount of free protoporphyrin can be calculated by difference. Thus, in iron deficiency anemia, we have found that up to 20% of the total porphyrin in circulating erythrocytes is protoporphyrin IX. In patients with sickle cell anemia who have hemoglobin type SS, up to 50% of the total porphyrin is free protoporphyrin IX. The presence of protoporphyrin IX is not related to the level of lead absorption, as it has been seen at both normal and elevated blood lead concentrations. In cord blood samples from newborn infants with elevated FEP, about one-third of the total porphyrin is protoporphyrin IX, while about two thirds is zinc protoporphyrin. Fractionation of cord blood into plasma, reticulocyte-rich and mature red blood cell fractions shows that mature red blood cells contain zinc protoporphyrin, while free protoporphyrin is concentrated in the reticulocyte-rich fraction containing very immature cells. These results are consistent with reports of others[4,8]; namely, free protoporphyrin is found in very immature erythrocytes which, in turn, are found in peripheral blood in the condition just described.

In conclusion, the data show that tests for FEP and zinc protoporphyrin will not always give equivalent results, even if protoporphyria, a rare genetic disorder, is excluded. Not only laboratory error, but also an increase in immature red cells can cause discrepancies between these tests. The new spectral data reported here for zinc protoporphyrin should further facilitate the standardization of various micro methods for its determination. Because of the presence of an interfering brown pigment in virtually all solid samples of zinc protoporphyrin, it is preferable to prepare zinc protoporphyrin standards freshly each day from protoporphyrin IX free base and zinc acetate. The concentrations of porphyrins in standard solutions are best determined from their absorptivity. To facilitate interlaboratory comparisons, appropriate absorptivity values should be selected and adhered to by all. Primary standardization must be made with zinc protoporphyrin and for the FEP method with protoporphyrin IX free base. If these and other factors are taken into account, greater consistency between results from different laboratories should result.

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