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Assays for Porphyrins, δ-Aminolevulinic-Acid Dehydratase, and Porphyrinogen Synthetase in Microliter Samples of Whole Blood: Applications to Metabolic Defects Involving the Heme Pathway

(lead poisoning/iron deficiency/uroporphyrin/coproporphyrin/protoporphyrin)

S. GRANICK, S. SASSA, J. L. GRANICK*, R. D. LEVERE*, AND A. KAPPAS

Rockefeller University, New York, N.Y. 10021; and *SUNY Downstate Medical Center, Brooklyn, New York 11203

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ABSTRACT **Three micromethods are described for the assay of enzymes or products of the heme biosynthetic pathway in blood. A fluorometric method for the assay of protoporphyrin may be applied to the rapid screening of children for chronic lead poisoning. A colorimetric assay for δ-aminolevulinic-acid dehydratase may be applied to the detection of acute and chronic lead poisoning. A fluorometric assay for porphyrin formation from porphobilinogen is also described.**

Several diseases, both hereditary and induced, cause changes in enzyme activities or in concentrations of intermediates in the heme biosynthetic pathway (1). We describe here three sensitive methods requiring aliquots of only 2–5 μl of whole blood per assay with which certain important alterations in this pathway may be determined.

In the first method, porphyrins in blood are determined by extraction and fluorescence measurement. The method is simple and useful for the detection of lead poisoning effects on erythroid cells during their maturation in the bone marrow, for the detection of iron deficiency anemia, and for the detection of the rare hereditary diseases of erythropoietic protoporphyria and congenital erythropoietic porphyria; in all of these disorders porphyrin concentrations in erythrocytes are elevated above normal (Table 1).

The second method is an assay for the enzyme δ-aminolevulinic-acid dehydratase. In lead poisoning, though not in iron deficiency anemia (2), a decreased activity of this enzyme is found in circulating erythrocytes (3). Thus, this method can distinguish between lead poisoning and iron deficiency. It is based on the conversion of δ-aminolevulinic acid by the enzyme to porphobilinogen (PBG), which is determined by the sensitive Ehrlich color reaction. In lead poisoning the -SH group of the dehydratase is affected, leading to a lowered activity of this enzyme.

The third method is an assay for the enzymatic conversion of PBG to porphyrins, primarily uroporphyrin by uroporphyrinogen synthetase. A low activity of this enzyme in liver has been claimed to be the direct cause of acute intermittent porphyria (1).

Determination of porphyrins in whole blood

Principle. 2 μl of capillary blood is transferred to a small test tube used as a fluorescence cuvette. Ethyl acetate–acetic acid 2:1 is added to solubilize the porphyrins. The porphyrins are extracted once—to the extent of 80%—with aqueous HCl. Without removal from the test tube, the aqueous phase is used directly for a fluorescence determination. The ratio of the two fluorescence peaks (605/655 nm) distinguishes between protoporphyrin and uroporphyrin or coproporphyrin. The small quantity of capillary blood required and the simplicity of the method make it readily applicable to rapid screening, for example, for lead poisoning in children.

A comprehensive study of porphyrin determinations in blood is that of Heilmeyer (6); a recent application for protoporphyrin fluorescence in lead poisoning is that developed by Whittaker Corp. (9). Our method is more sensitive and quantitative, avoids fluorescence quenching due to heme, and provides a ready identification between protopophyrin and other porphyrins.

Collection of Blood. Blood from a finger is drawn into an about 50-μl microhematocrit capillary containing heparin, (Clay Adams, Inc. N.Y.) and ejected into a disposable 1.5-ml polyethylene tube with cap (Bel Art Lab.). This blood is placed immediately on ice, as a routine procedure. Preservation on ice or in liquid N_2 is essential for the determination of dehydratase (method II), but is not necessary for the determination of porphyrins or synthetase.

Extraction of Blood. Pipette 2 μl of whole blood with a Drummond microdispenser into a disposable 1-ml glass test tube (6.5 × 50 mm, Rhesusröhrchen, Forma-Vitrum, Ltd. Switzerland). Rapidly add 300 μl of ethyl acetate–glacial acetic acid 2:1 with a Schwarz–Mann 1-ml Biopette. Then add 300 μl of 0.5 N HCl and mix with a vibrator mixer for 3 sec. Let the two phases separate for 5–10 min, or centrifuge. The bottom aqueous phase contains the porphyrins, the upper phase contains heme. Under these conditions, the aqueous phase will extract 86% of uroporphyrin, 83% of coproporphyrin, and 80% of protoporphyrin (Table 2).

Determination of Porphyrins by Fluorometry. A Hitachi–Perkin Elmer MPF-2A spectrofluorometer is used. The test tube is placed in the micro-cell holder of the instrument and scanned from 560 to 680 nm with 400-nm excitation light. For rapid analysis of porphyrins, a simple fluorometer (Turner model 111) has also been used. About 20–30 samples can be processed per hour.

Abbreviations: AmLev, δ-aminolevulinic acid; dehydratase, δ-aminolevulinic-acid dehydratase; PBG, porphobilinogen; synthetase, uroporphyrinogen synthetase.

TABLE 1. *Microassay for porphyrins, AmLev-dehydratase and porphyrinogen synthetase in blood: possible applications to detection of some diseased states*

Assay	Finding		Disease	μg/100 ml erythrocytes	Confirmatory test	Refs.
Porphyrins	Protoporphyrin	Normal	Normal	(20–50)		4
		Decreased	Megaloblastic anemia	(15–30)	Low serum Vitamin B-12	4
		Increased	Infection	(50–300)		4
			Increased erythropoiesis (hemolytic anemias)	(40–150)	Decreased erythrocyte survival and bone marrow M/E	4
			Thalassemia		Target cell, no abnormal Hb	5
			Sideroachrestic anemia (acquired)	(50–700)	Siderocytes, increased tissue iron	6
			Iron deficiency anemia	(200–800)	Low serum Fe, increased Fe-binding capacity, decreased tissue Fe	4
			Lead poisoning	(200–2000)	Lead determination, low dehydratase, decreased osmotic fragility	4*
			Erythropoietic protoporphyria	(2200–4400)	Photosensitivity, inheritance (dominant)	6
	Coproporphyrin	Normal	Normal	(0.5–2.3)		5
		Increased	Sideroachrestic anemia	(0–70)	Siderocytes, increased tissue iron	6
			Congenital erythropoietic porphyria	(90–125)	Type I porphyrins	6, 7
			Erythropoietic protoporphyria	(135–938)	Type III porphyrins	6
			Erythropoietic coproporphyria		Type III porphyrins	5
	Uroporphyrin	Normal	Normal	(trace)		5
		Increased	Congenital erythropoietic porphyria	(280–420)	Type I porphyrins	6, 7
			Erythropoietic protoporphyria	(10–96)		6
				nmol PBG/ml erythrocytes per hr		
AmLev-dehydratase activity		Normal	Normal	(320–900)		*
		Decreased	Lead poisoning	(40–140)	Lead determination	*
		Increased	Increased erythropoiesis		Increased ferrokinetics and reticulocytes	8
			Iron deficiency		Low serum Fe, increased Fe-binding capacity Decreased tissue iron	2
				nmol/ml erythrocytes per hr		
Porphyrinogen (uro- & coproporphyrinogen) synthetase activity		Normal	Normal	(35–50)		*
		Decreased	Acute intermittent porphyria		Neurological symptoms, AmLev, PBG in urine (dark urine particularly on standing)	14
		Increased	Hemolytic disorders		Decreased erythrocyte survival, increased osmotic fragility	

* Manuscript in preparation.

Porphyrin Values for Normal and Lead-Poisoned Erythrocytes. The porphyrin assayed is protoporphyrin, as determined by the criteria noted below. Normal values for protoporphyrin are 51.4 ± 8.4 (mean ±SD) μg/100 ml of erythrocytes. In individuals poisoned with lead the values range from 222 to 1098 μg/100 ml. The regression line correlating

log of protoporphyrin concentration with the blood lead concentration has a correlation coefficient of 0.72. For individuals whose bone marrow lead is in equilibrium with circulating lead, the correlation coefficient is 0.91, demonstrating the dependence of erythrocyte protoporphyrin concentration on the lead concentration in the bone marrow (manuscript in preparation).

Adjustment of the Spectrofluorometer. Slit widths of 20 nm are used for the monochromators of both the emitted and excited light in order to obtain maximum sensitivity. The excitation monochromator is set at 400 nm. For the excitation beam, a BG-2 and BG-4 filter (Jena Glaswerk Schott and Gen.) combination is used to transmit 400-nm light. For the emission beam, a Corning filter 2-63 is used to cut off light below 590 nm. A red-sensitive photomultiplier, R-136 tube, is used as the detector.

A standard fluorescence solution is used to adjust the instrument to a constant value or number of fluorescence units. A unit is defined as 0.01 division of the full-scale recording on a 23-cm chart at the maximal sensitivity. The standard is a Rhodamin B solution, 40 ng/ml in ethylene glycol. Under our conditions, the fluorescence produced gives an excursion of 347 units at 600 nm. The fluorometer is standardized each time the lamp has been turned on and becomes stabilized; it remains constant during a day's run.

Some Characteristics of the Porphyrins Assayed. Porphyrins in acid solution have two fluorescence bands, with maxima at about 605 and 655 nm. Although the intensity is higher for the 605-nm band, contaminants affect this band more readily than that at 655 nm. The intensity of the 655-nm emission is directly proportional to the concentration of porphyrin, and extrapolates to zero; the intensity of this band is used for quantitative determinations. For calibrations of the porphyrins and their extractability, solutions of uroporphyrin-I, coproporphyrin-III, and protoporphyrin-9 were used that had been chromatographed and crystallized in our laboratory; the concentrations of the standards were determined by absorption spectrophotometry in the Soret region, based on values reported by Rimington (10). The fluorescence units from 1-μM acidic porphyrins are reported in Table 2.

Distinction Between Protoporphyrin and the Other Porphyrins. An important characteristic of the porphyrins in acid solution is the ratio of the two fluorescence band maxima. Under our conditions for protoporphyrin, the intensity ratio for 605/655 nm is 2.05; for uro- it is 1.28, and for copro- it is 1.23. A ratio less then 2.05 is thus indicative of the presence of porphyrins other than protoporphyrin. The fluorescence band maxima also differ, coproporphyrin having maxima about 7-nm less, and uroporphyrin about 4-nm less, than protoporphyrin.

The presence of uro- and coproporphyrin may be indicative of certain erythrocytic porphyrias (Table 1). If their presence is suggested, two extractions are useful to discriminate between them and obtain a semiquantitative estimation. The following procedure is useful if uroporphyrin is the main component. A 2-μl aliquot of whole blood is pipetted into a 1-ml test tube and solubilized with ethylacetate–acetic acid 2:1, followed by extraction of the neutral porphyrins with 300 μl of 0.6 N NaOH. This procedure extracts into the aqueous phase 80% of uro-, and less than 10% of copro- and 1% of protoporphyrin. The single-band fluorescence maxima for neutral porphyrins are 624 nm for uro-, 622 for copro-, and 630 for protoporphyrin. 1 μM porphyrins dissolved in the components of the aqueous phase give about 36,000 fluorescence units for uro-, 44,000 units for copro-, and 24,000 units for protoporphyrin.

TABLE 2. *Fluorescence characteristics of acid porphyrins and their extractability from whole blood*

Porphyrin	Band maxima, nm λ_1	Band maxima, nm λ_2	Band minimum, nm	Ratio λ_1/λ_2	FU* λ_2 per 1 μM	Recovery, %
Proto	608	658	642	2.08	15,600	80
Uro	605	653	636	1.28	32,800	86
Copro	603	650	632	1.23	39,200	83

Whole blood (2 μl) is mixed in a 1-ml test tube with a known amount of standard porphyrin solution, and is extracted with 300 μl of ethylacetate–acetic acid 2:1, then with 300 μl of 0.5 N HCl. The fluorescence of the lower aqueous phase is determined.

* *FU*, fluorescence units.

The following procedure is useful to determine uro- and coproporphyrin. A 2-μl aliquot of whole blood is solubilized with ethylacetate–acetic acid 2:1; 300 μl of 0.05 N HCl is added and mixed vigorously. About 80% of uro-, 75% of copro-, and only 10% of protoporphyrin in their acidic forms are extracted into the aqueous phase. If little uroporphyrin is present, the fluorescence may then consist primarily of coproporphyrin, with maxima at 603 and 650 nm; the coproporphyrin concentration may be calculated from the values in Table 2.

Assay of AmLev dehydratase in whole blood

The method is essentially that of Mauzerall and Granick (11), modified for small amounts of enzyme. Weissberg *et al.* (12) have most recently reported on the use of this method as a sensitive index of subclinical lead poisoning. We have made the assay more sensitive by adjusting the pH of the reaction mixture, thus permitting the use of less blood, and by observing the restoration of enzyme activity with dithiothreitol (manuscript in preparation).

Principles of the Method. Lead poisoning is indicated by (*a*) low activity of the enzyme and (*b*) the complete restoration of enzyme activity when the incubation is in the presence of 20 mM dithiothreitol. Severe iron deficiency may cause an increase of about 50% in the activity of the dehydratase above normal (2).

Stability of Dehydratase Activity. It is imperative to cool the blood sample in an ice bath as soon as possible after collection to prevent loss of SH groups from the enzyme. When heparinized blood is stored at 4°, the activity is constant for 24 hr, decreases by 15% on the fifth day, and by 30% on the 12th day. No activity is lost when the blood is stored in liquid N_2, but 50% of the activity is lost by day 2 if blood is stored at −20° under air. The loss of activity is not due to enzyme degradation, for activity is restored completely by the addition of dithiothreitol.

Method. Three sets of 0.3-ml polyethylene microtubes with captive plugs are prepared, each containing 5 μl of

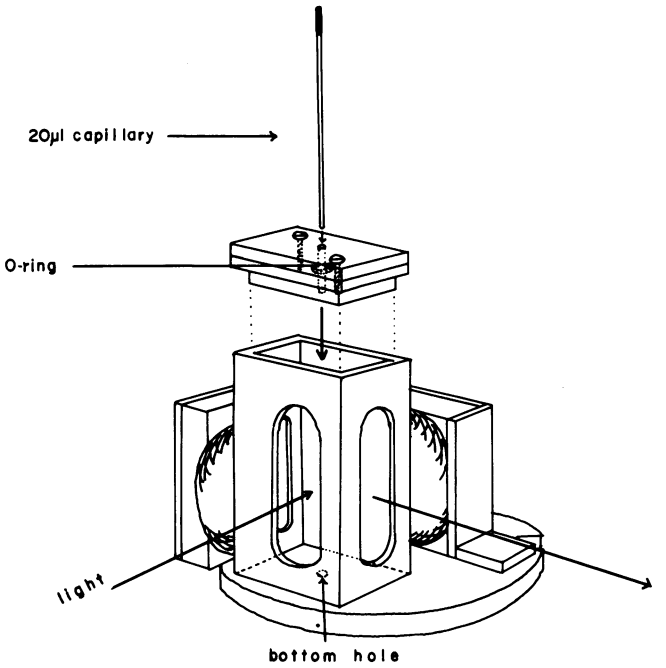

FIG. 1. High sensitivity fluorescence cell holder adapted as capillary holder.

heparinized whole blood at 0–4°; the tubes are placed in an ice bath.

(*i*) In the tissue control set, 5 μl of 0.1 M NaH_2PO_4–Na_2HPO_4 (pH 5.8)–1.25% saponin is added.

(*ii*) In the reagent set, 5 μl of buffer–saponin containing 670 μg/ml of of AmLev · HCl is added.

(*iii*) In the reagent + SH set, 5 μl buffer–saponin containing 670 μg/ml of AmLev · HCl plus 3.1 mg/ml of dithiothreitol is added.

The final pH of these mixtures is 6.3. The mixture is vibrated for 3 sec, and the tubes are incubated at 37° for 1 hr in a water bath. The reaction is terminated by the addition of 200 μl of trichloroacetic–$HgCl_2$ solution (4 g of Cl_3CCOOH + 2.7 g of $HgCl_2$ per 100 ml), then centrifuged at 1000 × *g* for 5 min. 150 μl is removed from the supernatant for determination of product PBG and mixed with 150 μl of the modified Ehrlich reagent; the absorbance is determined between 10–15 min. in a Cary 15 spectrophotometer in a 1-cm cell, 4-mm wide, with a mask 3-mm wide × 2.5 mm high. The colored solutions are measured against the control set in the reference compartment. The spectrum is recorded from 650 to 450 nm. The molar absorption coefficient of the Ehrlich salt at 553 nm is 6.1×10^4 (11). The ratio of the two absorption bands at 553/525 nm should be 0.85 (11).

The modified Ehrlich-Hg Reagent. To 168 ml of glacial acetic acid add 40 ml of 70% perchloric acid, 4.0 g of recrystallized *p*-dimethylaminobenzaldehyde, and 0.7 g of $HgCl_2$ (dissolved in a small amount of water), and dilute with water to 220 ml. This reagent is usable for a week or longer if kept at 4°. Freshly prepared reagent is less colored.

In Lead Poisoning, i.e. a concentration of lead in blood greater than 60 μg/100 ml, dehydratase activity is in the range of 41–320 nmol of PBG per hr per ml of erythrocytes at 37°; in normal blood, dehydratase activity is in the range of 600–895 (mean of 620). The logarithm of dehydratase activity is inversely correlated with blood-lead concentration, with a correlation coefficient of 0.76, in agreement with results of Weissberg *et al.* (12).

Assay of porphyrin formation from porphobilinogen: activity of synthetase

Principles of the Method. To conserve costly PBG, 10 μl is incubated to form porphyrins; with this volume the results are not appreciably affected by heme quenching.

Method. In a 0.3-ml polyethylene microtube with captive microcap, 5 μl of whole blood is quickly mixed with 5 μl of 0.1 M phosphate buffer (pH 7.4) containing 1.25% saponin and 488 μg/ml of PBG (2 mM). The mixture is incubated at 37° for 1 hr in a water bath in the dark. The reaction is stopped by addition of 25 μl of ethylacetate–acetic acid 2:1, mixed rapidly, and then extracted by mixture with 25 μl of 0.5 N HCl. The tube is stoppered and centrifuged in a hematocrit centrifuge (Clay–Adams, Inc. N.J.) for 1 min to separate the two phases. For fluorescence determination, a 20-μl Drummond microcap is used as a reproducible disposable cuvette. The capillary is inserted into the aqueous phase and filled almost completely with the extract; the upper end of the capillary is sealed with Crito-seal. The capillary is placed in a high-sensitivity cell holder, (Hitachi–Perkin Elmer), and accurately positioned as shown in Fig. 1. The sealed end of the capillary is inserted through the bottom of a Lucite plate fitted with an "O" ring. The lucite plate is placed snugly on the top of the cell holder, and the capillary is pushed down to fit into a small hole at the bottom of the cell holder. Fluorescence is measured as described in method I. The recovery of porphyrins is 84% for uroporphyrin, 81% for coporporphyrin, and 80% for protoporphyrin. The fluorescence intensity at the longer-wavelength emission band for a 1-μM solution is 3300 for uroporphyrin, 3520 for coporporphyrin, and 1540 for protoporphyrin.

Calculations for Porphyrins Formed by Incubation of Hemolysate with PBG. Since most of the porphyrin formed under these conditions is uroporphyrin, with some coproporphyrin, the amount of porphyrin formed may be calculated as uroporphyrin. The amount can also be considered as the sum of uro- plus coproporphyrin with an error less than 15%, since the fluorescence intensity and the recovery of these two porphyrins in the aqueous phase are similar (Table 2). With the capillary method, λ_{max} is displaced about 6 nm to a shorter wavelength.

Comments

The incubation of PBG with blood hemolysate results in the formation mainly of uroporphyrin, with some coproporphyrin. Since the extraction of both uroporphyrin and coproporphyrin are similar and the fluorescences are also similar and the isomers—whether I or III—are indistinguishable, the fluorescence may be reported in terms of the sum of uroporphyrin plus coproporphyrin.

A 1 mM PBG concentration is used as standard substrate. Porphyrin formation from PBG is linear for at least 2 hr at 37°. The nonenzymic condensation of PBG to form fluorescent compounds is less than 5% of the total fluorescence resulting from the enzyme reactions. In a 2-hr incubation, about 10% of the PBG is consumed (as determined colori-

metrically with the Ehrlich reagent); of this amount, 95% is converted to porphyrin as determined by fluorometry.

Although the enzymes produce porphyrinogens, the extraction procedures indicate that the porphyrinogens are all autoxidized to porphyrins by the time they are measured in the fluorometer. Exposure to ultraviolet light or addition of I_2 to the extract does not increase the fluorescence. Extraction with 1.5 N HCl is to be avoided, because some heme will become dissolved in the aqueous solution; upon exposure to the UV light of the fluorometer, iron can be released from the heme, thus causing an artifactual increase of porphyrins.

The enzymes converting PBG to porphyrin are stable for many weeks when stored at 4°. The enzymes are not affected by Pb^{++}, Hg^{++}, *p*-chloromercuribenzoate, *N*-ethylmaleimide, iodoacetamide, sodium tetrathionate, EDTA, or dithiothreitol at concentrations of about 0.1–1 mM.

DISCUSSION

The micro-methods described here for the content in erythrocytes of protoporphyrin, the activity of δ-aminolevulinic-acid dehydratase, and porphyrin formation from porphobilinogen are sufficiently sensitive so that all three may be determined in duplicate on a single blood sample totaling 40 μl. The sensitivity of these methods permits their application to large population studies of acquired or inherited defects in heme biosynthesis. In addition, because of their sensitivity, these assays may be applied to small amounts of tissue, such as those available by biopsy.

In Table 1, we summarize the kinds of acquired and inherited lesions that may be studied by these methods. For example, the use of the protoporphyrin assay for lead screening may also aid in the identification of rare genetic diseases such as the erythropoietic porphyrias, as well as more common disorders such as iron deficiency anemia.

We have shown how two of the methods may be specifically applied to the detection of lead poisoning. These methods complement each other. The assay for protoporphyrin will indicate chronic lead exposure; this is so because in bone marrow, protoporphyrin is formed in the mitochondria of maturing erythrocytes. The mature erythrocytes are devoid of mitochondria and lack the ability to make protoporphyrin. The concentration of protoporphyrin in blood is thus a reflection of its impaired conversion to heme in the presence of lead in bone marrow. Protoporphyrin, however, is not only elevated in lead poisoning, but it is also elevated in iron deficiency anemia. To distinguish between these two common problems, the cytosol enzyme δ-aminolevulinic-acid dehydratase, may be assayed. In lead poisoning, the activity of this enzyme will be decreased, whereas in iron deficiency it will either be normal or elevated. In contrast to the protoporphyrin assay, the assay for δ-aminolevulinic-acid dehydratase reflects lead effects in circulating erythrocytes, as well as in bone-marrow erythroid cells; it may, therefore, indicate either acute or chronic lead intoxication. Thus, by performing both assays one may be able not only to distinguish acute from chronic lead poisoning, but the presence of iron deficiency anemia as well.

The recent development by Delves (13) of a rapid sensitive method for the determination of lead in blood by atomic absorption spectrophotometry will also permit the detection of lead exposure, which may either be acute or chronic. Our protoporphyrin method for whole blood is somewhat simpler, equally as rapid, and permits categorizing the lead exposure as being chronic. By far the largest number of cases we have examined belong to this category. It may prove useful to have analyses by both methods to indicate the recency of exposure to this metal, and to recognize iron deficiency anemia.

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