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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF PROTOPORPHYRIN AND ZINC PROTOPORPHYRIN IN BLOOD

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SUMMARY

Zinc protoporphyrin and protoporphyrin free acid in blood were determined by high-performance liquid chromatography on a C_{18} column. Results for 63 human blood samples obtained through a lead poisoning detection program compared favorably with the widely-used ethyl acetate—acetic acid extraction determination. Blood from 16 rats which had been maintained on water heavily spiked with chloroform or bromodichloromethane and blood from a lead-poisoned cow were examined by this procedure.

INTRODUCTION

It is difficult to determine any of the specific porphyrins in blood (Table I) because so many other absorbing and fluorescing species are present. Interlaboratory agreement for protoporphyrin (PP) in blood is lacking, probably because of the many spectroscopic interferences and the lack of a good primary standard [1]. To be accurate a method must be capable of efficiently separating the porphyrin of interest not only from the other porphyrins but also from these interferences.

Most laboratories which assay blood samples to detect lead poisoning measure porphyrins routinely. However, they must perform double-solvent extractions in order to separate protoporphyrin from blood and hemin [2]. Many laboratories use a hematofluorometer, which measures porphyrin fluorescence directly from whole blood and therefore relies solely on optical filters for porphyrin specificity [3].

High-performance liquid chromatography (HPLC) has been successfully applied to the analysis of porphyrins [4—6]. Esterified 2-8-carboxylic acid-substituted prophyrins can be easily separated by adsorption on a 20-cm, 5-μm Partisil

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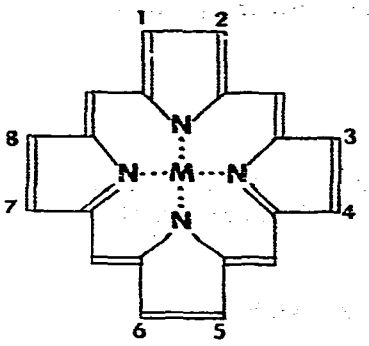
TABLE I

STRUCTURES OF SEVERAL PORPHYRINS IN HUMAN BLOOD



Porphyrin	M^{2+}	Substituents on positions		
		1,3,5,8	2,4	6,7
Uroporphyrin III	2H	$-CH_2COOH$	$-CH_2CH_2COOH$	$-CH_2CH_2COOH$
Coproporphyrin III	2H	$-CH_3$	$-CH_2CH_2COOH$	$-CH_2CH_2COOH$
Protoporphyrin IX	2H	$-CH_3$	$-CHCH_2$	$-CH_2CH_2COOH$
Zn protoporphyrin IX	Zn	$-CH_3$	$-CHCH_2$	$-CH_2CH_2COOH$
Fe protoporphyrin IX (heme)	Fe	$-CH_3$	$-CHCH_2$	$-CH_2CH_2COOH$

column with a solvent such as ethyl acetate—cyclohexane (60:40) [7]. However, the added esterification step is time-consuming, especially when large numbers of samples must be analyzed. Ion-pair separation of free acids has been reported [8]. This approach was rejected for the present work because of the added complexity which its use would introduce with regard to formation of the zinc complex.

We have developed a rapid HPLC method for accurate determination of porphyrin free acids in blood using a C_{18}-bonded column packing. Using a primary standard which has been assayed by this method, we have determined PP and zinc protoporphyrin (ZnP) in blood and compared the results with those obtained by conventional methods.

EXPERIMENTAL

Apparatus

A modified Waters Assoc. (Milford, Mass., U.S.A.) high-performance liquid chromatograph was used in the isocratic mode. The individual components included a Waters 6000A solvent delivery system, a Rheodyne 7105 continuous-flow septumless injector (Perkin Elmer, Norwalk, Conn., U.S.A.), a Waters 30 cm × 4 mm I.D. reversed-phase μBondapak C_{18} column (10-μm particle size), and a Dupont (Wilmington, Del., U.S.A.) Model 836 fluorescence detector (medium-pressure Hg source, R777 PMT) equipped with a λ_{ex} = 365-nm filter (Corning CS-7-60, Corning, N.Y., U.S.A.) and a λ_{em} = 595-nm filter (source unknown).

A Hewlett-Packard 3385A data system was used for all measurements. Integration was improved by shunting a 1-mF capacitor to the data system input.

A Dupont Sorvall Superspeed SS3 centrifuge equipped with an SS-34 rotor was used to separate the cellular debris.

Reagents

Methanol and ethyl acetate were purchased from Burdick and Jackson Labs. (Muskegon, Mich., U.S.A.), acetic acid and zinc acetate from J.T. Baker (Phillipsburg, N.J., U.S.A.).

Uroporphyrin, coproporphyrin, protoporphyrin, and zinc protoporphyrin are separated (Fig. 1) using the mobile phase methanol—acetic acid—deionized water (83:2:15) (pH 3.9) which has been degassed by vacuum filtration through a 0.22-μm Teflon filter (Millipore, Bedford, Mass., U.S.A.). Blood samples, however, contain large amounts of hemin, which obscures the earlier peaks; therefore a more nonpolar 39:4:7 (pH 3.4) solvent mixture was chosen to speed up the analysis time for ZnP and PP.

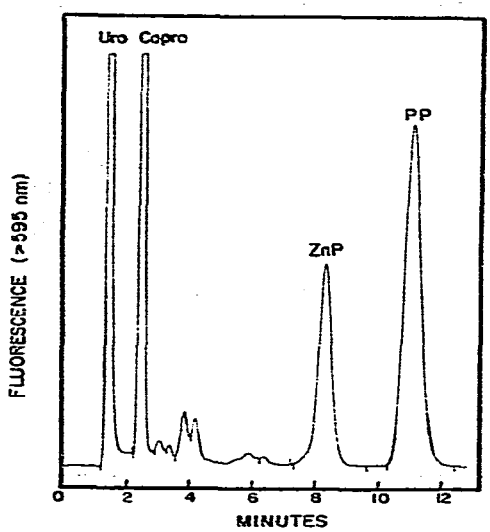


Fig. 1. HPLC separation of porphyrin free acids: uroporphyrin, coproporphyrin, ZnP, and PP. Sample, 100 μl of porphyrin standards (0.1—0.5 μg/ml) in the mobile phase; instrument, modified Waters; column, μBondapak C_{18} 10 μm; mobile phase, methanol—acetic acid—water (83:2:15); flow-rate, 2 ml/min; temperature, ambient; detector, Dupont 836 fluorescence, λ_{ex} = 365 nm, λ_{em} >595 nm.

A stock disodium protoporphyrin (Na_2PP) standard was prepared by dissolving 13.7 mg of the anhydrous salt (Sigma, St. Louis, Mo., U.S.A.) in 1 l of acetone—water (7:3) solvent. This standard was corrected for purity (see Discussion).

Dilute working standards were prepared daily as follows: for PP, 0.5 ml of the Na_2PP stock solution was diluted to 100 ml with chromatographic solvent. For ZnP, 3 ml of the Na_2PP stock solution, 3 ml of 0.3% zinc acetate in 1% acetic acid, and 50 ml of methanol were reacted for 1 h in a 100-ml volumetric

flask, then diluted to obtain a solvent composition of approximately 89:4:7.

The solvent used to extract porphyrins from blood was ethyl acetate—acetic acid (4:1).

Samples

Sixty-three human blood samples were chosen at random from among those submitted to this Divison's lead poisoning detection program.

Rat blood was obtained from Nya:NYLAR rats by drawing from the tail. The rats had been maintained for over 2 years on a water supply containing chloroform or bromodichloromethane (2 ml/l).

Cow blood was obtained from a cow which is regularly fed lead acetate for the production of quality-control samples for the New York State lead poisoning detection program.

All blood samples were collected into glass microcapillaries or Vacutainers (Becton Dickenson, Rutherford, N.J., U.S.A.), containing disodium ethylene dinitrilotetraacetic acid (EDTA) and stored away from light at 4°.

The freeze-dried pooled human blood was purchased from A.R. Smith Labs., Los Angeles, Calif., U.S.A.

Procedure

With an Eppendorf pipette 200 μl of blood are added to 100 μl of water in a glass centrifuge tube. The porphyrins are extracted by addition of 1 ml of ethyl acetate—acetic acid (80:20), followed immediately by vigorous mixing. The sample is then centrifuged for 1 min at 17,369 *g*, and 25 μl of the porphyrin-containing supernatant are injected onto the column.

The standards are injected separately with no extraction. Total analysis time for ZnP and PP is about 15 min/sample.

Chromatographic conditions

To minimize adsorption of hemin and porphyrins onto any polar silicic acid sites, the C_{18} column must first be washed with 50 ml of 0.01 *N* HCl. ZnP, PP, and hemin in blood extracts are then well separated as free acids in 5 min at ambient temperature, using the methanol—acetic acid—water solvent at a flow-rate of 2 ml/min. Between sample injections a 5-min washout time of sample residues is required, otherwise significant porphyrin adsorption will occur.

RESULTS AND DISCUSSION

Because it minimizes spectroscopic interferences, this HPLC method may be applied to many matrices containing porphyrin. However, blood samples were of particular interest because of their routine use for clinical assays.

Human, bovine, and rat blood samples were analyzed (Fig. 2) and found to contain varying amounts of ZnP and PP. Although about 90% of the protoporphyrin in whole human blood is found present as the Zn^{2+} complex, about 10% is not complexed. The 4-8-COOH-porphyrins may not be seen in the chromatograms because of hemin absorbance from 1.5 to 2.5 min, but an extraction solvent which eliminated hemin (possibly ethanol [9]) would permit analysis of these porphyrins.

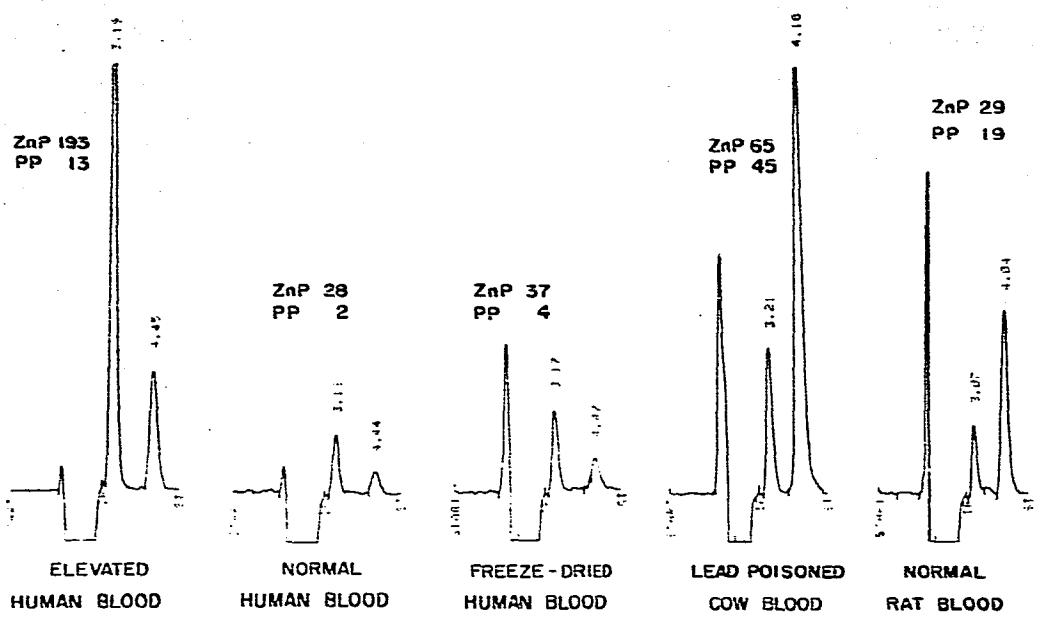


Fig. 2. Chromatograms of ZnP (3.1 min) and PP (4.4 min) for several different kinds of blood. Amounts are in μg/dl.

Sixty human blood samples were analyzed by HPLC for total protoporphyrin (ZnP + PP), commonly called erythrocyte protoporphyrin (EP). These samples were also analyzed by three established methods: for lead by a microsampling-cup atomic absorption method [10], for ZnP by a commercial filter hematofluorometer with fluorescent-dye calibration slides, and for total protoporphyrin (PP + ZnP) by a double-extraction fluorescence method [2]. These three established methods are routine in our laboratory, and our performance is checked routinely by participation in several interlaboratory surveys. A summary of these results is given in Table II.

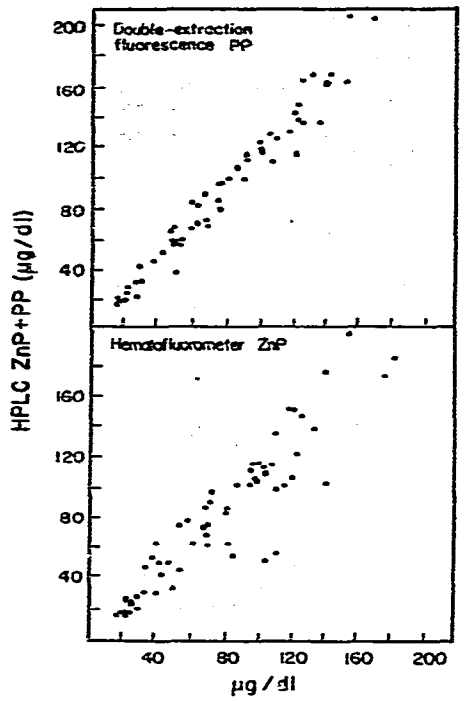
The lead content of samples ranged from 9 to 63 μg/dl, total porphyrin content ranged from 18 to 204 μg/dl.

TABLE II

CORRELATION OF RESULTS FOR 60 BLOOD SAMPLES

R^2 = Correlation coefficient squared, indicates the proportion of the population represented by the regression [11]; b_1 = slope of line and b_0 = intercept on Y-axis.

Independent variable (X)	Dependent variable (y)	R^2	b_1	b_0
Double-extraction ZnP + PP	HPLC ZnP + PP	0.96	1.1	1.7
Double-extraction ZnP + PP	HPLC ZnP	0.96	0.97	1.8
Double-extraction ZnP + PP	Hemat. ZnP	0.88	0.85	6.5
Hemat. ZnP	HPLC ZnP	0.87	1.0	4.1
Hemat. ZnP	HPLC ZnP + PP	0.86	1.1	4.9
Double-extraction ZnP + PP	HPLC PP	0.43	0.11	−0.36
HPLC ZnP	HPLC PP	0.38	0.11	0.38
Hemat. ZnP	HPLC PP	0.37	0.11	0.42



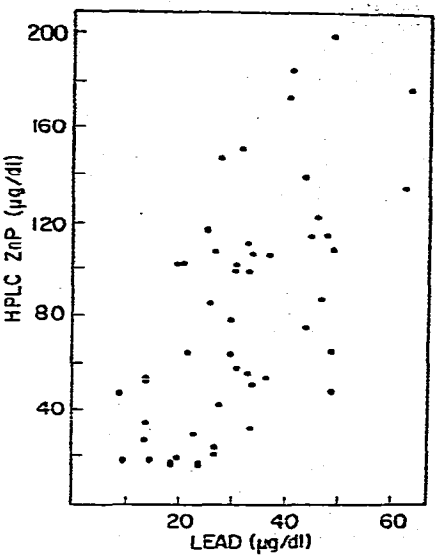


Fig. 3. Comparison of results of actual samples analyzed by HPLC and either the double-extraction fluorescence method or a hematofluorometer.

Fig. 4. Comparison of results of actual samples analyzed for ZnP by HPLC and for lead by microsampling-cup atomic absorption.

Consistently good agreement was found between HPLC and double-extraction fluorescence results for total porphyrins (Fig. 3). Hematofluorometric results correlated less well, but free uncomplexed protoporphyrin did not really correlate with the other measurements at all. Clearly the proportion of zinc-complexed to uncomplexed protoporphyrin is variable and may reflect the trace zinc content of the subject.

Because of our interest in detection of subclinical lead poisoning, we investigated the correlation between blood lead and HPLC ZnP results. In view of the good agreement between the HPLC ZnP + PP and double-extraction fluorescence ZnP + PP, it seems likely that the very poor correlation between blood lead and HPLC ZnP (Fig. 4) is physiological and not a product of methodology.

To obtain good quantitative results with the C_{18} columns currently available, the pH of the column, mobile phase, and sample must be controlled. The retention time of hemin was very sensitive to column acidity, requiring deactivation of the column by washing with strong acid. With predominantly hydrophobic packing, the best separation for uroporphyrin, coproporphyrin, ZnP, and PP was found (Fig. 1) as expected, when the mobile phase was maintained slightly below the isoelectric point of the porphyrins (pH 4—5). At significantly higher and lower pH values, porphyrin ionization caused a drastic loss of resolution. Injection of standards or samples containing less than 2% acetic acid is not re-

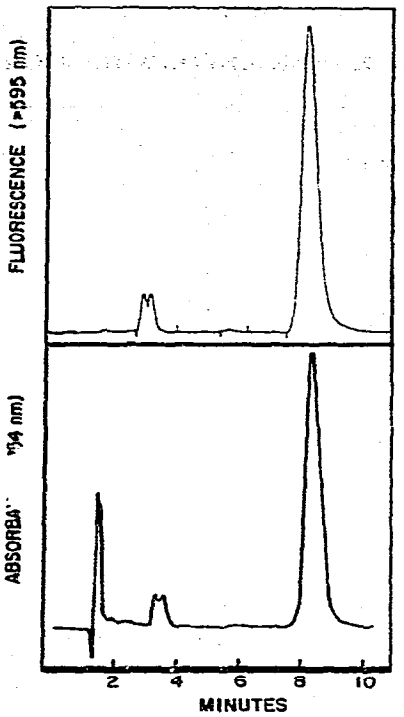


Fig. 5. Chromatogram of impure disodium protoporphyrin IX primary standard.

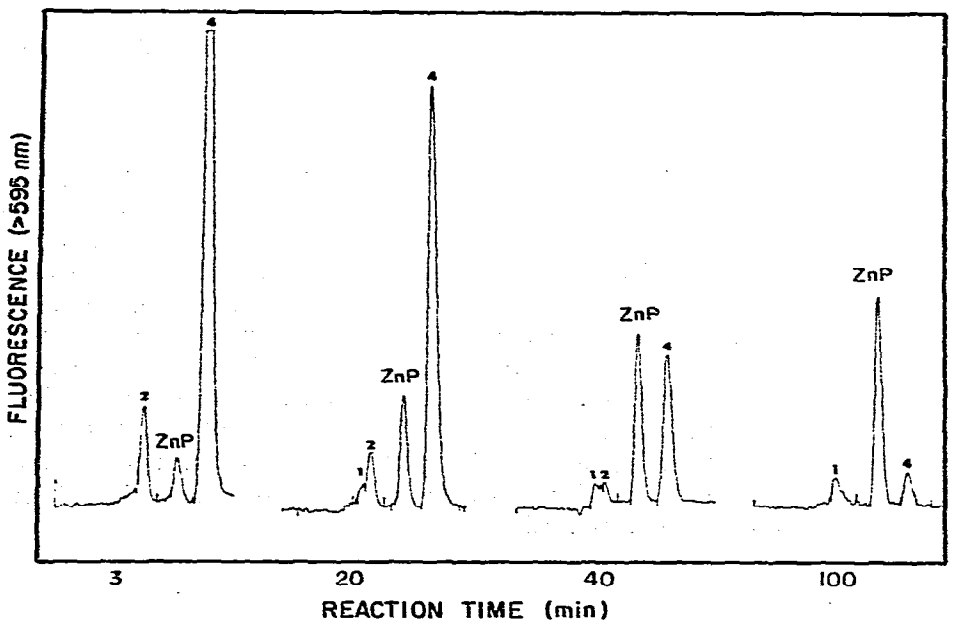


Fig. 6. Reaction of Zn^{2+} with protoporphyrin IX standard in methanol. Peaks: 1 = Zn complex of an unknown porphyrin impurity; 2 = unknown porphyrin impurity; 3 = ZnP; 4 = disodium protoporphyrin IX starting material.

TABLE III

PORPHYRIN (μg/dl) IN THE BLOOD OF RATS FED EITHER CHLOROFORM OR BROMODICHLOROMETHANE DAILY FOR 2 YEARS

$CHCl_3$		$CHBrCl_2$		Controls	
ZnP	PP	ZnP	PP	ZnP	PP
23	12	24	18	25	12
21	15	36	15	25	10
21	12	32	16	28	13
38	23	21	9	28	16
29	19	30	13	32	13
27	16	22	14	30	12
35	16	29	11	24	11
–	–	29	9	29	10
–	–	21	12	26	16
–	–	–	–	28	9
Mean					
27.7	16.1	27.1	13.0	27.5	12.2

commended, as up to 30% of the porphyrin may be lost by column adsorption. The Na_2PP IX standard was found to contain two small impurities (9% by area) by absorbance at 254 nm and by red fluorescence detection (Fig. 5). Efforts were made to prepare ZnP from Na_2PP and to remove the impurity by fractional crystallization, but Zn complexes of the impurities always appeared in the product (Fig. 6). Hence it was assumed that the impurities were porphyrin in nature, and the concentration of Na_2PP stock solution was corrected for 91% purity. Preparation of a working ZnP standard by reaction of the Na_2PP stock solution with Zn^{2+} in methanol (Fig. 6) yielded a ZnP standard which was stable for 1 day.

A number of instances have been reported in the literature where exposure to chlorinated organic compounds has produced hepatic porphyria. Administration of hexachlorobenzene [12], polychlorinated biphenyls [13], or 2,3,7,8-tetrachlorodibenzo-1,4-dioxin [14] produced hepatic porphyria in some mammals, explained by a decrease in the activity of decarboxylation enzymes. Currently we are conducting a chronic toxicology study with chloroform and bromodichloromethane in rats. Thus far an effect has not been reported in erythrocytes, and so the analysis was applied to blood samples from rats exposed for two years to these compounds at approximately 20 mg per kg body weight per day. Samples from rats exposed for two years to either chloroform or bromodichloromethane were analyzed for ZnP or PP by HPLC. Table III shows clearly that no effect on levels of ZnP or PP has been produced. More likely porphyrias, such as those found by San Martin de Viale et al. [15] in the liver or kidney, have not yet been studied.

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