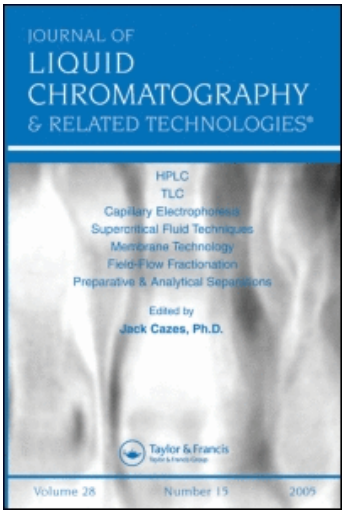

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DETERMINATION OF PORPHYRINS IN HUMAN BLOOD BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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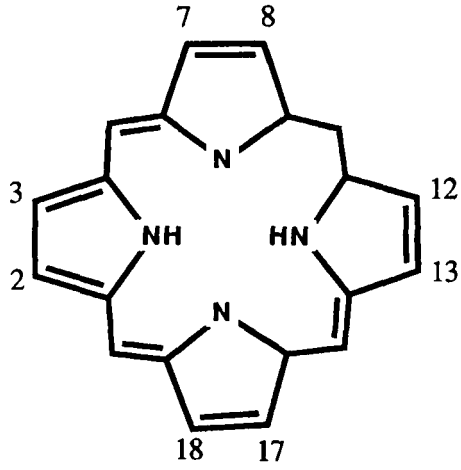
ABSTRACT

A method for the reversed-phase high performance liquid chromatography (HPLC) of porphyrin carboxylic acids and zinc protoporphyrin with fluorescence detection is described. The method is applied to determine the porphyrins in human blood. Erythrocyte porphyrins were extracted from whole blood with 300 µl of acidic methanol solution. Metalloporphyrin was separately extracted with an acetone-pyridine-sterox solution from another aliquot of blood. The supernatants from both extracts were simultaneously injected onto a µBondapak C18 column for detection of porphyrins with excitation wavelength at 405 nm and emission wavelength at 630 nm. The separation required a pre-treatment of the column with a binary mobile phase containing 0.1 M sodium phosphate in acetonitrile (29 : 16, v/v, pH 5.3) for 10 min prior to sample injection. The separation was completed isocratically by increasing the acetonitrile concentration in the mobile phase (0.1 M sodium phosphate : acetonitrile = 18 : 129, v/v, pH 5.3) 4 min after injection. The elution of all the compounds took less than 23 min.

INTRODUCTION

Biological polycarboxylic porphyrins and zinc protoporphyrin are intermediate metabolites of heme biosynthesis, which starts by the condensation reaction between the two precursors, δ- aminolevulinic acid (ALA) and porphobilinogen (PBG) to form the first porphyrins. The metal-free porphyrins are the immediate precursors of other porphyrins and , subsequently, the various hemes. Disturbances in heme biosynthesis are characterised by the elevation of porphyrins and the precursors in body fluids and tissues (1). Determination of porphyrins in human blood is important in diagnosis of lead poisoning, iron deficiency anemia and different kinds of porphyrias. Analysis of accumulated and excreted porphyrins and metalloporphyrins could serve as a confirmatiory test for distinguishing lead poisoning from iron deficiency anemia, which shows strikingly similar biochemical and clinical symptoms. High performance liquid chromatography has become the preferred analytical technique because it allows accurate determination of porphyrins very efficiently and offers excellent sensitivity for detection of picomole of porphyrins from biological specimens using fluorometry. However, simultaneous determination of biological porphyrins presents a challenging problem due to their structural similarity (Table I) and the chemical stability of zinc-protoporphyrin in acidic solutions. Numerous chromatographic methods for the determination of porphyrins have been reported (2-14). More recently, studies dealing with the retention behavior of the polycarboxylic porphyrins on the reversed-phase chromatography (15), standard materials for quantification of porphyrins (16), and several improved separation methods for the determination of porphyrins by liquid chromatography

TABLE I Structures of Porphyrins



Porphyrins	Substituents* at Position							
	2	3	7	8	12	13	17	18
Uroporphyrin	A	P	A	P	A	P	A	P
Heptaporphyrin	M	P	A	P	A	P	P	A
Hexaporphyrin	M	P	M	P	A	P	P	A
Pentaporphyrin	M	P	M	P	A	P	P	M
Coproporphyrin	M	P	M	P	M	P	M	P
Protoporphyrin	M	V	M	V	M	P	P	M
Mesoporphyrin	M	E	M	E	M	P	P	M
Zn-protoporphyrin	M	V	M	V	M	P	P	M

* M= $-CH_3$; E= $-C_2H_5$; A= $-CH_2COOH$; P= $-CH_2CH_2COOH$; V= $-CH=CH_2$

(16-18) have appeared. A high-speed reversed-phase HPLC method using an octadecylsilyl 3 cm column to separate selected porphyrin isomers (19), and cyclodextrin bonded phases with a novel mobile phase to isocratically separate the major biological porphyrins have also been described (20). The solution chemistry of porphyrins during analysis has been reported recently as well (21). The desirability for the simultaneous determination of porphyrins and zinc-protoporphyrin in blood prompted the development of a HPLC method .

The present paper describes two simple extraction methods for porphyrin carboxylic acids and zinc-protoporphyrin in human blood to facilitate a reversed-phase HPLC method with fluorometric detection for the simultaneous determination of the compounds.

EXPERIMENTAL

Materials

Methanol and acetonitrile of HPLC grade were purchased from J.T. Baker Chemical Co. (Phillipsburg, NJ). Sterox solution was obtained from Banco Anderson Labs. (Fort Worth, TX). Porphyrin acid chromatographic marker , zinc-protoporphyrin and protoporphyrin IX were the products from Porphyrin Products, Inc. (Logan, UT). All other chemicals were of reagent grade.

Instrumentation

The chromatographic system consisted of a Varian Model 5000 Liquid Chromatograph (Varian Associates, Palo Alto, CA) fitted with a Rheodyne

7126 injector with a 200-µl sample loop , and a Perkin-Elmer Model 650-15 variable wavelength spectrofluorometer (Perkin-Elmer, USA) equipped with a 12-µl flow cell. The analytical column was a µBondapak C18 column (3.9 mm x 30 cm) from Waters Associates (MA, USA). A guard column from Whatman was used to protect the analytical column. Results were reported as relative fluorescence. A Hewlett-Packard 3388A integrator was used for all measurements. All pH readings were taken on a Model 601 digital ionalyzer with a Ross combination pH electrode purchased from Orion Research (MA, USA).

Extraction of zinc-protoporphyrin and porphyrin carboxylic acids

Zinc-protoporphyrin and porphyrin carboxylic acids were extracted from whole blood according to the previous procedures with modifications (12). Briefly, liquid blood (50 µl) was vortex-mixed with 350 µl of an extraction solution containing acetone, pyridine and sterox soltuion (20 : 1 : 1, v/v/v, respectively) for 5 min. The mixture was spun down at 2500 x g for 5 min. The supernatant was collected for the analysis by HPLC.

The extraction of porphyrin carboxylic acids was achieved by vortex-mixing another 50 µl of blood with 350 µl of acidified methanol (1.5 M HCl : methanol, 1 : 2, v/v) for 5 min. The supernatant was collected after centrifugation at 2500 x g for 5 min. The supernatants from both extracts were simultaneously injected onto the HPLC for the analyis of zinc-protoporphyrin and porphyrin carboxylic acids in a single run.

Preparation of standards

Porphyrin standard stock solutions were prepared by dissolving the contents of the chromatographic marker standard in 1 M HCl. The zinc-protoporphyrin and protoporphyrin IX standards were dissolved in a minimum amount of pyridine and diluted to different concentrations with methanol. The standard solutions were stable when stored at 4°C.

Chromatographic conditions

The procedures were carried out following the previously reported methods with modifications (18). In brief, a two-step separation of zinc-protoporphyrin and porphyrin carboxylic acids was performed. The column was pre-treated with the binary mobile phase containing 0.1 M sodium phosphate in acetonitrile (29 : 16, v/v, pH 5.3) for 10 min following by sample injection. The mobile phase was switched to the second binary mobile phase with higher concentration in acetonitrile (18 : 129, v/v, pH 5.3) 4 min after injection. The elution was performed at a flow rate of 1.1 ml/min. The eluent was monitored at 405 nm for the excitation wavelength and 630 nm for the emission wavelength.

Recovery study

The recovery of each compound was determined in triplicate using the internal standard method previously reported (12). Blood samples were fortified with measured amounts of porphyrin standards.

RESULTS AND DISCUSSION

The isocratic separation of the biological porphyrin carboxylic acids and the internal standard, mesoporphyrin using conventional chromatography technique is very challenging , and has never been reported before. Inclusion of zinc-protoporphyrin in the separation process results in additional difficulty due to co-elution and stability of the compounds. However, the analysis of accumulated porphyrins and metalloporphyrins in body fluids is so important in diagnosis of different porphyrin-related disorders that an efficient method for simultaneous assay of these compounds could be very useful for clinical testing. Liquid chromatography is the preferred technique ; thus, the mobile phase pH, composition and elution strength of a traditional LC method were studied . The results were discussed earlier (15). A good resolution between porphyrin peaks is dependent on the pH of the mobile phase. A pH of 5.3 is optimal to minimize demetallation of zinc-protoporphyrin , and produces an acceptable resolution between peaks. However, a two-step elution of porphyrin carboxylic acids, zinc-protoporphyrin and the internal standard, mesoporphyrin , is required to completely separate the compounds. A representative chromatogram for the separation of the eight porphyrin standards is shown in Figure 1. The elution strength of the mobile phase was increased in the second mobile phase to completely elute the less polar porphyrins, namely, zinc-protoporphyrin, mesoporphyrin and protoporphyrin , within 25 min. The pre-treatment of the column with a mobile phase of weaker elution strength is important in ensuring a good resolution among the more polar porphyrin carboxylic acids. The separation

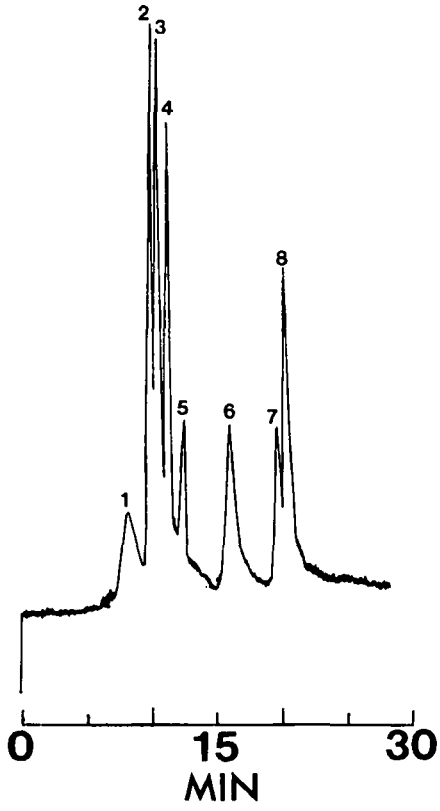


Figure 1 Chromatogram of zinc-protoporphyrin and porphyrin carboxylic acid standards. Chromatographic conditions are given in the text. Peaks: 1, uroporphyrin; 2, heptaporphyrin; 3, hexaporphyrin; 4, pentaporphyrin; 5, coproporphyrin; 6, zinc-protoporphyrin (0.04 nmol); 7, mesoporphyrin(0.02 nmol); 8, protoporphyrin (0.05 nmol); other peaks contain 0.02 nmol of the corresponding compounds.

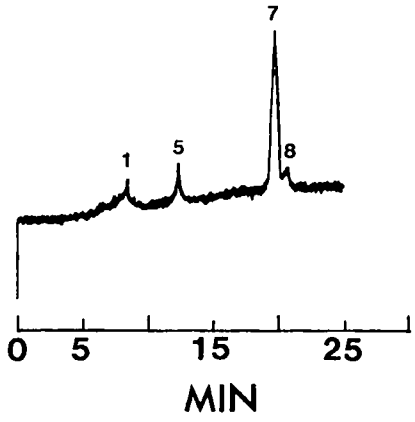


Figure 2 A typical chromatogram of blood sample from a normal individual. See Figure 1 for experimental conditions and labels.

efficiency was maximized at a constant flow rate of 1.1 ml/min throughout the elution. An acceptable resolution between peaks , as shown in Figure 1, could only be achieved with the optimal flow rate, elution strength of the mobile phase and the optimal period of the pre-treatment process. Despite a two-step elution, the results were very reproducible. The retention of the porphyrin carboxylic acids increases as the number of carboxyl groups decreases. The elution order of the dicarboxylated porphyrins, mesoporphyrin and protoporphyrin acids, resembles that on the reversed-phase C18 column, with the more polar zinc-protoporphyrin eluting before mesoporphyrin followed by protoporphyrin acids.

Application of the method to blood samples from normal individuals was carried out . A typical elution profile of erythrocyte porphyrins from a normal blood specimen is shown in Figure 2. In human lead-poisoning, protoporphyrin also exists as zinc-protoporphyrin. Therefore, the method

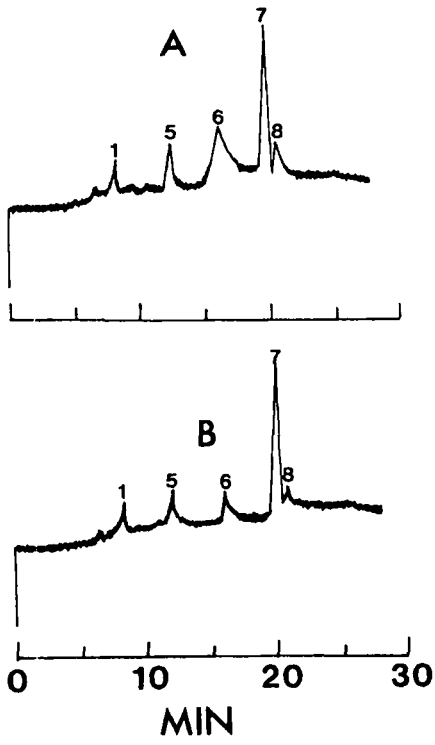


Figure 3 Chromatograms of blood samples from (A) patient #1 and (B) patient #2. See Figure 1 for experimental conditions and labels.

was applied to blood samples from two patients diagnosed with lead poisoning. The chromatograms of porphyrins from patients' blood specimens are shown in Figure 3. The chromatograms indicate that there are no interfering peaks throughout the elution. The detection limit is in the subpicomole for the porphyrins. The method offers excellent sensitivity for determining porphyrins in 50 µl of whole blood. Although the assay requires two different extractions , the analysis of these compounds can be

TABLE II Reproducibility and Recovery in Analysis of Porphyrins in Blood

	Relative standard deviation(C.V. %) day-to-day precision[a]	Average Recovery (%)[b]
Uroporphyrin	12.0	91
Heptaporphyrin	9.8	95
Hexaporphyrin	8.2	96
Pentaporphyrin	9.1	94
Coproporphyrin	8.2	96
Mesoporphyrin	7.2	97
Protoporphyrin	10.3	95
Zn-protoporphyrin	11.1	90

[a] Average value from four runs.
[b] Average of triplicate runs of blood samples.

performed simultaneously by the HPLC method in the same run. However, the acid extraction results in demetallation of zinc-protoporphyrin . Consequently, the acidified methanol extraction procedure facilitates quantification of total erythrocyte porphyrins concentration by the present method ; thus, the protoporphyrin peak contains protoporphyrin and zinc protoporphyrin. The acetone-pyridine-sterox extraction technique is very

TABLE III Concentrations of Porphyrins in Blood from Four Normal Individuals and Two Patients (μg/dl)*

	Uroporphyrin	Coproporphyrin	Protoporphyrin	Zn-Protoporphyrin
NORMAL				
1.	4.3	4.2	28.2	ND
2.	4.0	3.7	30.3	ND
3.	3.4	2.3	29.5	ND
4.	5.2	3.2	25.3	ND
PATIENT				
1.	7.2	5.3	46.2	15.5
2.	5.6	4.5	40.1	8.1

ND, not detected.
* Average of duplicate runs of blood sample.

efficient and quite specific for zinc-protoporphyrin. The efficiency of the method was evaluated and summaried in Table II. The recovery study of these compounds shows an excellent accuracy of quantification with an efficiency over 90 percent for the compounds. The major erythrocyte porphyrin acids in a normal individual are uroporphyrin, coproporphyrin and protoporphyrin. There are no other detectable porphyrins in blood from

a normal individual. However, an additional porphyrin, zinc protoporphyrin, is present in both patients only. The results (Table III) are in good agreement with the previous studies cited in the references in the present paper. For clinical testing , it is necessary to run more blood specimens from different age groups and subjects for statistical analysis so as to establish a possible cut-off for individual porphyrins. Nevertheless, the presence of the major porphyrins determined by the present method are consistent with the literature.

In conclusion, the method permits a fast diagnosis of porphyrin related disorders by measuring individual porphyrin acids in blood. Determination of accumulated zinc-protoporphyrin can be performed simultaneously within the same run , and could provide a definitive test for lead-poisoning. The method is simple and reliable, and can be applied to study porphyrin acids in other biological tissues.

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