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SEPARATION OF PORPHYRIN ISOMERS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-speed reversed-phase high-performance liquid chromatographic method using an octadecylsilyl 3 cm long (3 μm particle size) column to separate the free acids of uroporphyrins I and III and coproporphyrins I and III from each other, and from the type I isomers of several other porphyrin carboxylic acids, is described. Separation of the porphyrins was achieved in less than 8 min, and injections were possible every 12 min. The detection limits of uroporphyrin, coproporphyrin, and mesoporphyrin were 75, 45, and 35 fmol (at a signal-to-noise ratio of 2), respectively. Application of the method to the determination of urinary and liver porphyrin patterns is shown.

INTRODUCTION

The porphyrias are disorders which result in excessive production and excretion of porphyrins. The disorders may be inherited, or they may be caused by exposure to certain toxicants [1, 2]. Understanding of the etiology of the porphyrias and the monitoring of populations exposed to porphyrogenic chemicals has been advanced by measuring the relative levels of the porphyrins (the 'porphyrin patterns') in excreta, blood, and tissue. During the past decade, high-performance liquid chromatography (HPLC) has become the preferred technique to determine porphyrin patterns. Many excellent normal- and reversed-phase methods have been published, and a recent review discusses the relative merits of each approach [3].

Although many HPLC methods can achieve excellent separation of porphyrins, very few of them can separate porphyrin isomers (e.g., uroporphyrin

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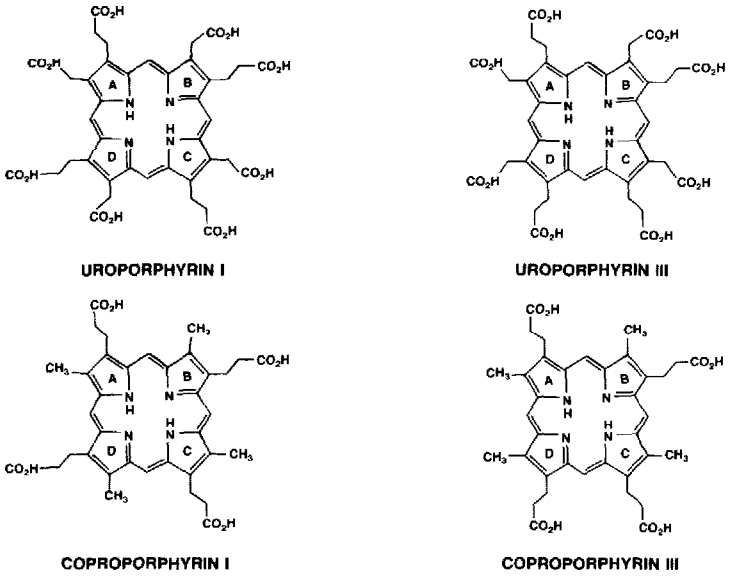


Fig. 1. Structures of uroporphyrins I and III and coproporphyrins I and III.

I from uroporphyrin III and coproporphyrin I from coproporphyrin III; Fig. 1). The only reversed-phase methods appear to be those of Lim and co-workers. This group published methods which could separate the free acids of uroporphyrins I and III [4] and coproporphyrins I and III [4, 5]. A method that achieved the separation of at least thirteen porphyrins in a single injection employed a 15 cm trimethylsilyl column (i.e., a C_1 column) and gradient elution with a mobile phase containing acetonitrile, methanol, and ammonium acetate buffer [6]. Although useful, the latter method suffers from several disadvantages. These include (1) the long chromatography time (the time between injections is approximately 1 h), (2) the use of expensive analytical columns, and (3) the use of acetonitrile (an expensive and toxic solvent). For these reasons we conducted studies to determine if the mobile phase conditions of Lim and co-workers [4, 5] could be adapted to high-speed HPLC and to determine if acetonitrile could be replaced with methanol. This paper presents the results of these studies.

EXPERIMENTAL

Reagents

Porphyrins (free acids) were obtained from Porphyrin Products (Logan, UT, U.S.A.). Hexachlorobenzene (97%) was from Aldrich (Milwaukee, WI, U.S.A.). Methanol and acetonitrile were from Anachemia (Montreal, Canada). Ammonium acetate (AnalaR or HPLC grade) was from BDH (Ville St.-

Laurent, Canada) or Fisher Scientific (Ottawa, Canada), respectively. Glacial acetic acid was from J.T. Baker (Phillipsburg, NJ, U.S.A.). Water was treated with a Milli-RO/Milli-Q purification system obtained from Millipore (Mississauga, Canada). All other reagents were of analytical grade and were purchased from Fisher Scientific.

Equipment

Concentrations of porphyrin standard solutions were determined with a Hewlett-Packard 8452A diode array spectrophotometer. An Ultra-Turrax homogenizer (Janke and Kunkel) and a Fisher Centrific centrifuge were used for tissue preparation. Disposable Sep-Pak C_{18} cartridges obtained from Waters (Mississauga, Canada) were used for sample concentration. HPLC was carried out with a Perkin-Elmer Series 4 liquid chromatograph equipped with a Rheodyne 7135 loop injector valve (5 μl capacity), a Perkin-Elmer 3 cm long C_{18} column (3 μm particle size), and a Perkin-Elmer LS-4 fluorescence spectrophotometer with the excitation wavelength adjusted to 365 or 400 nm (15 nm slit) and the emission wavelength set at 624 nm (20 nm slit).

Chromatographic conditions

Gradient elution of porphyrins was achieved with an ammonium acetate–methanol mobile phase. An ammonium acetate solution (1 *M*, adjusted to pH 5.2 with glacial acetic acid) was filtered through a 0.45-μm filter prior to use. The flow-rate was 2.0 ml/min, and injections were made when the mobile phase was methanol–ammonium acetate (30:70, v/v). A linear gradient, which was commenced upon injection, brought the composition to 45:55 in 1 min. A second linear gradient then raised the methanol concentration to 90% in 4 min. This composition was held for 2 min, and a reverse gradient returned the mobile phase to the starting composition in 0.5 min.

Preparation of porphyrin standard solutions

Mixed standard solutions containing the type I isomers of uroporphyrin, hepta-, hexa-, penta-, and coproporphyrin, and mesoporphyrin IX were prepared from the porphyrin acid chromatographic marker kit by dissolving 10 nmol of each in 1 ml of 12 *M* hydrochloric acid and by diluting to 1 *M* with water. Solutions of types I and III isomers of uroporphyrin and coproporphyrin, and mesoporphyrin IX were prepared by dissolving approximately 500 μg of the porphyrin(s) in 1 ml of 12 *M* hydrochloric acid and diluting with water to obtain 1 *M* hydrochloric acid solutions. Millimolar extinction coefficients [7] of 505 (406 nm), 470 (401 nm), and 390 (401 nm) were used to determine uroporphyrin, coproporphyrin, and mesoporphyrin concentrations, respectively. Dilutions of these stock solutions were prepared with 1 *M* hydrochloric acid.

Sample preparation

Hepatic porphyria was induced in female Wistar rats (Charles River) by providing them with a diet containing hexachlorobenzene (1000 ppm). Details are reported elsewhere [8]. Similarly, hepatic porphyria was induced in female Japanese Quail, *Coturnix coturnix japonica* (obtained from Deschambeault Agricultural Research Institute, Deschambeault, Canada) by providing them with a diet containing hexachlorobenzene (50 ppm). After six weeks of feeding upon the contaminated diets, the animals were sacrificed and their livers were removed. Porphyrins were extracted from the livers which 0.9 *M* perchloric acid–methanol (50:50, v/v) and concentrated with C_{18} Sep-Pak cartridges using the previously described method [9].

Preparation of human urine samples for HPLC analysis was similar to the procedure previously reported [10]. Urine samples (24 h) from normal and porphyric patients (provided by S. Perkins, Division of Biochemistry, Civic Hospital, Ottawa, Canada) were collected in 5 g/l sodium carbonate and stored at −20 °C. For analysis, 2-ml aliquots of urine were acidified to pH 1.0 with 6 *M* hydrochloric acid, filtered through 0.45-μm filters, and injected directly into the HPLC system.

RESULTS AND DISCUSSION

Fig. 2 shows the separation of several commercially available porphyrin standards. Baseline resolution of all type I isomers and near baseline resolution of uroporphyrins I and III and of coproporphyrins I and III was achieved in less than 8 min. Protoporphyrin (not shown in this chromatogram) eluted approximately 0.4 min after mesoporphyrin. Allowing for column re-equilibration, injections were possible every 12 min. The degree of resolution is similar to that attained by Lim and Peters [6], but it was achieved in approximately one sixth of the time. Variance in porphyrin retention times was less than 3%.

The detection limits (signal-to-noise ratio of 2) of uroporphyrins I and III, coproporphyrins I and III, and mesoporphyrin IX were 75, 45, and 35 fmol, respectively, with the detector excitation wavelength adjusted to 365 nm (15 nm slit) and the emission wavelength adjusted to 624 nm (20 nm slit). Sensitivity was approximately two times higher with the detector excitation wavelength set at 400 nm, and interference by methanol contaminants was not observed at this wavelength. (We have previously reported that contaminants in some batches of methanol can interfere with porphyrin analysis when the excitation wavelength is set at 400 nm [9].

Table I shows the peak height reproducibility of the method for repeated injections of several porphyrins. At concentrations which approached the limits of detection, the coefficients of variation (C.V.) for six injections of uroporphyrins I and III, coproporphyrins I and III, and mesoporphyrin IX ranged from 11 to 16%. At concentrations that were from four- to five-fold higher, the

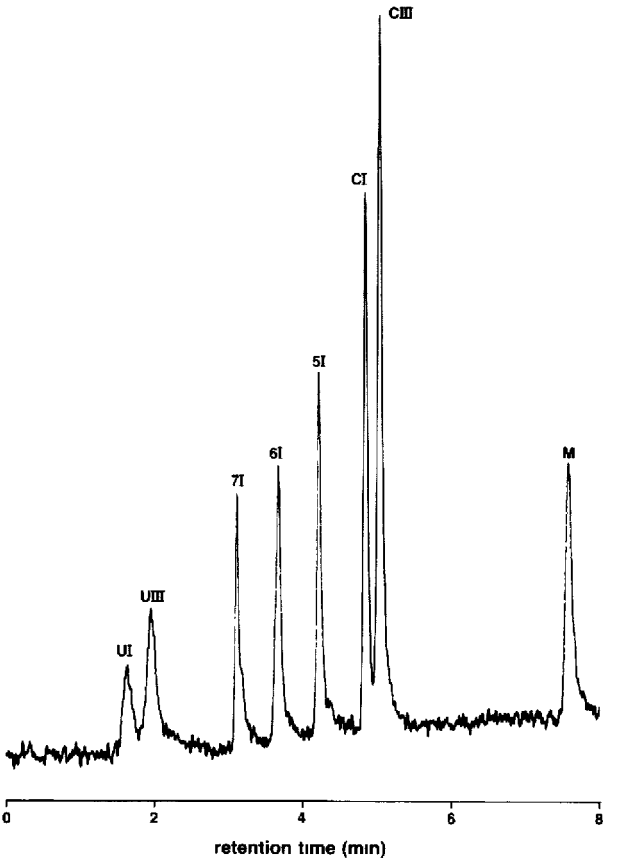


Fig. 2. Separation of a solution of porphyrin carboxylic acid standards (in 1 *M* hydrochloric acid) containing approximately 500 fmol per injection of uroporphyrins I and III (UI and UIII), coproporphyrins I and III (CI and CIII), mesoporphyrin IX (M), and the type I isomers of hepta-, hexa-, and pentacarboxylic acid porphyrins (7I, 6I, 5I). Fluorescence detector settings: sensitivity, fixed scale, 25; excitation, 365 nm (15 nm slit); emission, 624 nm (20 nm slit).

reproducibility was substantially improved for the coproporphyrins and for mesoporphyrin (3 and 4%, respectively) but it ranged from 9 to 13% for the uroporphyrins. It is possible that slightly longer re-equilibrium times between injections would improve the reproducibility of the uroporphyrin peak heights.

The method was tested with extracts of the livers of Wistar rats and Japanese Quail with hexachlorobenzene-induced porphyria, and Fig. 3 shows typical chromatograms. Uroporphyrins I and III were detected in both species, and the analysis of several samples showed uroporphyrin III to be seven to ten-fold more concentrated then uroporphyrin I. The presence of uroporphyrin III is thought by most researchers to be caused by inhibition of uroporphyrinogen decarboxylase [11], but the cause of elevated uroporphyrin I is unknown. Each

TABLE I

REPRODUCIBILITY OF PEAK HEIGHTS OF REPEATED INJECTIONS OF SEVERAL PORPHYRIN STANDARDS ($n=6$)

Porphyrin	Concentration (pmol per 5 μl)	Peak height (mm)	Coefficient of variation (%)
Uroporphyrin I	0.23	23.7	15.6
Uroporphyrin III	0.23	27 3	12.8
Coproporphyrin I	0.08	15.0	11.3
Coproporphyrin III	0.07	17.0	12.3
Mesoporphyrin IX	0.11	11.8	14.4
Uroporphyrin I	1.15	115.8	13.5
Uroporphyrin III	1.04	126.5	9.4
Coproporphyrin I	0.37	93.2	2.8
Coproporphyrin III	0.35	85.3	2.7
Mesoporphyrin IX	0.46	60.3	4.0

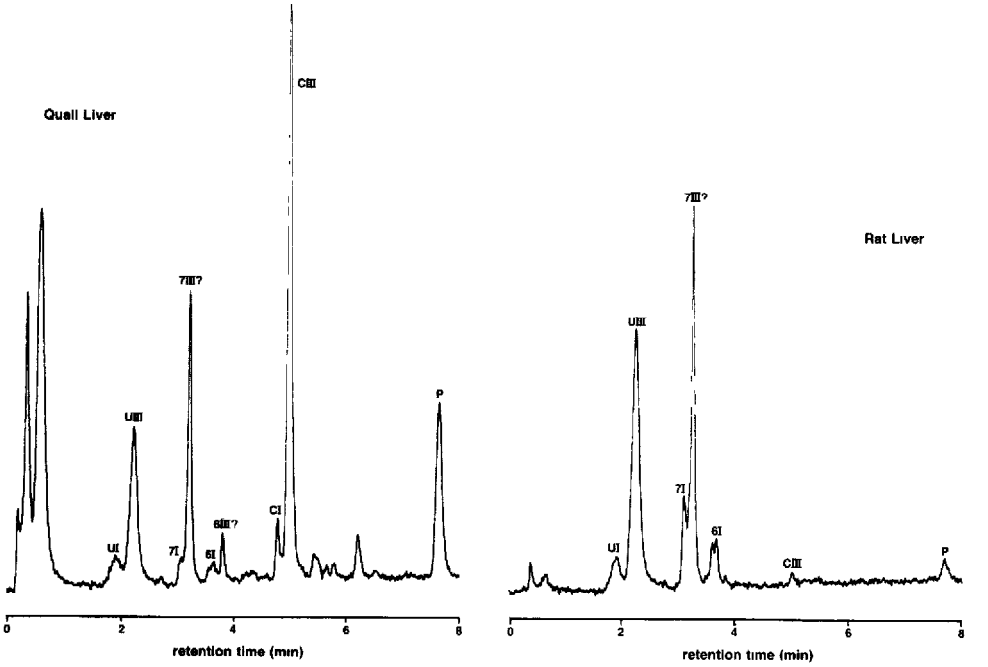


Fig. 3. Illustration of the porphyrin patterns in extracts of livers of Japanese Quail and Wistar rats with hexachlorobenzene-induced porphyria. Peak identification is as in Fig. 2; P = protoporphyrin. Fluorescence detector settings: sensitivity, fixed scale, 10; excitation, 365 nm (15 nm slit); emission, 624 nm (20 nm slit).

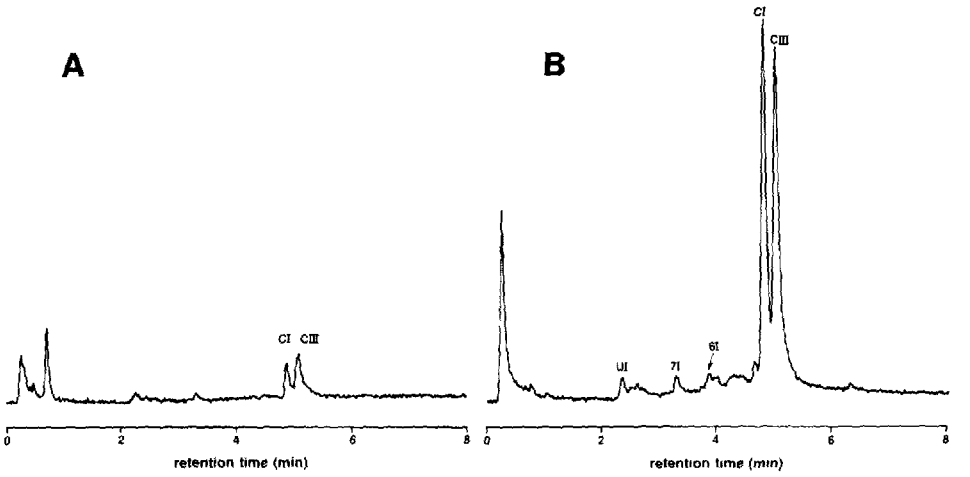
chromatogram shows small peaks which co-elute with heptacarboxylic acid porphyrin I followed by large peaks of what is likely heptacarboxylic acid porphyrin III. Unfortunately, heptacarboxylic acid porphyrin III is not commercially available (nor are the type III isomers of hexa- and pentacarboxylic acid porphyrins), and the identity of the latter peaks is not certain. However, in the methods of Lim and co-workers [4–6], the type III isomers elute after the type I isomers, and this likely occurs in the present method as well. Peaks which eluted with hexacarboxylic acid porphyrin I were found in both species. Coproporphyrins I and III were well resolved (shown for quail liver, but not for rat liver).

Fig. 4 shows the urinary porphyrin patterns from a normal individual, a patient with porphyria variegata, and a patient with porphyria cutanea tarda. As previously shown by others [3, 6], both coproporphyrin I and III were present in normal urine. The urine of the patient with porphyria variegata had approximately equivalent amounts of coproporphyrins I and III, and the urine of the porphyria cutanea tarda patient contained approximately 1.5 times more uroporphyrin I than uroporphyrin III. Each chromatogram shows several unidentified peaks. While it is likely that the unidentified peaks are porphyrins, confirmation (by fluorescence spectra) was not obtained.

The peak which co-eluted with the heptacarboxylic acid porphyrin I in the urine of the porphyria cutanea tarda patient is intriguing. Other investigators have stated that heptacarboxylic acid III is characteristic of this disease [6, 12, 13]. Either the presently described method is unable to separate the types I and III isomers of this porphyrin, or this particular patient has an uncharacteristic form of porphyria cutanea tarda.

Lim and co-workers [4, 6] concluded that it was not possible to resolve uroporphyrin I and III isomers with methanol as the organic modifier on C_1, C_8, or C_{18} columns, and they suggested that this was likely due to excessive hydrogen bonding of the eight carboxylic acid groups of uroporphyrin to methanol adsorbed on the stationary phase. Replacement of methanol with acetonitrile (a non-hydrogen bonding organic modifier) resulted in excellent resolution of the isomers. However, the work presented here shows that methanol was able to separate the isomers extremely well on the 3 cm long C_{18} columns. This finding supports the conclusion reached by other researchers (see, for example refs. 14 and 15) that the development of new HPLC methods can benefit with the testing of reversed-phase columns obtained from different manufacturers. It is not always possible to predict with accuracy the chromatographic behavior of compounds on a particular column. Differences due to varying amounts of 'carbon loading', 'end-capping', and particle size and shape are among the factors which can influence reversed-phase chromatography.

Over the past several years we have discovered that the Perkin-Elmer C_{18} columns are generally excellent for separating porphyrins [9, 10]. However, some lots of columns have been completely ineffective. Interestingly, these



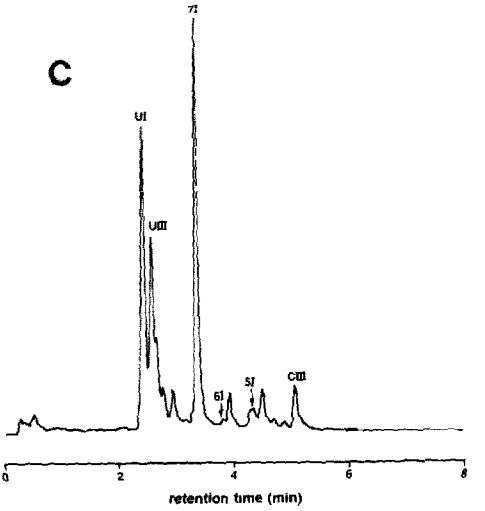


Fig. 4. Chromatograms which illustrate the separation of porphyrin isomers in normal urine (A) and in the urine of patients with porphyria variegata (B) and porphyria cutanea tarda (C). Peak identification is as in Fig. 2. Fluorescence detector setting: sensitivity, fixed scale, 0.5; excitation, 400 nm (15 nm slit); emission, 624 nm (20 nm slit).

columns were not defective per se as they passed the quality assurance test of the manufacturer (they could separate a standard mixture of aromatic compounds). It was hypothesized that the reason for the poor chromatography of porphyrins was due to inadequate end-capping of some batches of packing. Unreacted silanols can greatly influence retention and selectivity, and this has been shown to be especially true for the separation of protonated amines due to absorption and/or ion exchange on the accessible silanols [15]. Extremely poor peak shape of the basic phenylthiohydantoin (PTH) derivatives of amino acids histidine and arginine on C_{18} columns that have not been end-capped is suggested to result from slow desorption kinetics associated with this reaction [15]. At the pH used for the chromatography of porphyrins the pyrrole nitrogens are protonated [16], and the explanation offered for amines and amino acids would therefore seem to apply to porphyrins. Perkin-Elmer [17] confirmed that a batch of columns which would not separate a mixture of porphyrins had not been thoroughly end-capped, thus providing support for the hypothesis. We therefore recommend that researchers who are interested in using our techniques ensure that the columns which they purchase have been end-capped. In our experience, columns manufactured by Supelco (Bellefonte, PA, U.S.A.) perform as well as those from Perkin-Elmer.

CONCLUSION

The free acids of several porphyrins have been separated within 8 min on a 3 cm long C_{18} (3 μm particle size) column using a gradient consisting of ammonium acetate and methanol. The method can be applied to the determination of the porphyrin patterns in urine and liver. Advantages of the method are (1) the short chromatography time, (2) the utilization of relatively inexpensive columns, and (3) the utilization of methanol rather than acetonitrile.

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