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Short Communication

Improved method to extract and concentrate porphyrins from liver tissue for analysis by high-performance liquid chromatography

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ABSTRACT

A new method to extract and concentrate porphyrins from liver tissue for analysis by high-performance liquid chromatography is described. Porphyrins were extracted with acetonitrile–hydrochloric acid, concentrated on disposable octadecylsilyl columns, brought to dryness, and reconstituted in 1.2 *M* hydrochloric acid. Dried porphyrins were stable at −80°C for at least three weeks, and porphyrins reconstituted in hydrochloric acid were stable for at least 20 h. Yields of uro-, heptacarboxyl-, hexacarboxyl-, pentacarboxyl- and coproporphyrin were ≥90%. The method allows determination of porphyrin concentrations in small (10–100 mg) samples of liver tissue.

INTRODUCTION

Porphyrins and porphyrinogens are cyclic tetrapyrroles that are intermediates of heme biosynthesis [1]. High-performance liquid chromatography (HPLC) with fluorescence detection is currently the most commonly used method to separate and quantify porphyrins extracted from biological tissues (for recent review articles, see refs. 2–4).

Perchloric acid–methanol is frequently used to extract porphyrins from whole liver tissues [5] and cultured hepatocytes [6]. We previously described a method for extracting porphyrins from biological tissues with perchloric acid–methanol. With this method, extracts were concentrated with disposable octadecylsilyl columns prior to analysis by HPLC [7,8]. Although perchloric acid–methanol allowed effective extraction of most porphyrins from tissues, we have observed that uro-, heptacarboxyl- and hexacarboxylporphyrin are relatively unstable in this solvent. In addition, there are undesirable hazards associated with perchloric acid because it can be explosive upon evaporation.

We have developed an improved extraction and concentration method to overcome problems associated with perchloric acid–methanol. Porphyrins were extracted with acetonitrile–HCl, concentrated on octadecylsilyl columns, brought

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to dryness under nitrogen and reconstituted in HCl. This paper describes the new method and compares it to the perchloric acid–methanol method.

EXPERIMENTAL

Reagents

Porphyrin standards (dihydrochlorides) were from Porphyrin Products (Logan, UT, USA). HPLC-grade methanol and acetonitrile were from Caledon Labs. (Georgetown, Canada). Water was treated with a Milli-RO–Milli-Q purification system obtained from Millipore (Mississauga, Canada). All other reagents were purchased from Fisher Scientific (Nepean, Canada). Chicken liver was obtained from a local supplier and Sprague–Dawley rat liver was provided by Health and Welfare, Canada.

Equipment

Porphyrins were extracted with a Wheaton overhead stirrer equipped with a Potter-Elvehjehm PTFE homogenizer. Centrifugation was carried out with a Sorvall Model RT6000B centrifuge. Porphyrins were concentrated on trifunctional (tC_{18}) Sep-Pak Plus cartridges (Waters, Mississauga, Canada) using a Sep-Pak cartridge rack connected to a Welch Model 1400 vacuum pump. A Bransonic Model 42 ultrasonicator was obtained from Baxter-Canlab (Mississauga, Canada). HPLC was carried out with a Perkin-Elmer Series 4 liquid chromatograph equipped with a Rheodyne 7125 loop injector valve, a Perkin-Elmer 3-cm-long C_{18} column (3 μm particle size), and a Perkin-Elmer LS-4 spectrofluorometer equipped with a Hamamatsu R928 red-sensitive photomultiplier tube.

Porphyrin extraction and concentration

Acetonitrile–HCl method. Most analyses were carried out with previously frozen liver. Frozen liver (10–100 mg) was added to homogenizing tubes, weighed and allowed to thaw. To each tube, 6 ml of HCl (1 *M*)–acetonitrile (1:1) were added. The tissue was homogenized using six up and down strokes with the homogenizer prior to centrifugation for 15 min at 1000 *g*. Supernatants were transferred to glass tubes, and pellets were extracted and centrifuged as described for the first extraction. Supernatants from second extractions were transferred to the tubes that contained the first extracts, and the solutions were diluted to 50 ml with water. The purpose of the dilution step was to modify the polarity of the solvent so that porphyrins would be retained on Sep-Paks. Sep-Paks were prepared by wetting them first with acetonitrile (10 ml) and then with water (15 ml). The extracts were concentrated on Sep-Paks and eluates were discarded. Porphyrins were eluted with acetonitrile (2.5 ml). Acetonitrile was evaporated under nitrogen, and samples were either prepared immediately for analysis by HPLC or were stored at −80°C. Dried extracts from samples that weighed from 50 to 100 mg were prepared for HPLC analysis with the addition of concentrated HCl (100 μl). The tubes were shaken by vortex for approximately 10 s and allowed to stand for 5 min. Water (900 μl) was added to each tube, the tubes were shaken by vortex for approximately 5 s, placed in the water bath of an ultrasonicator, and sonicated for approximately 5 min. Dried extracts from samples that weighed 10 mg or less were prepared using the same procedure as that described for larger samples except that the volumes of concentrated HCl and water were 10 and 90 μl, respectively. All solutions were filtered through 0.45-μm filters prior to injection onto the HPLC column.

Perchloric acid–methanol method. Several experiments were carried out to compare the acetonitrile–HCl method with the previously described perchloric acid–methanol method [7]. Perchloric acid (0.9 *M*)–methanol (6 ml) was added to liver tissue (100 mg), and homogenization was carried out using six up and down strokes with the homogenizer prior to centrifugation for 15 min at 1000 *g*. Supernatants were transferred to glass tubes, and pellets were extracted and centrifuged as described for the first extraction. Supernatants from the second extractions were transferred to tubes which contained the first extracts and the solutions were diluted to 50 ml with water. Sep-Paks were prepared for concentrating porphyrins

by wetting them first with methanol (10 ml) and then with water (15 ml). Extracts were concentrated on Sep-Paks and eluents were discarded. Porphyrins were eluted with methanol (2.5 ml) and filtered (0.45 μm) prior to analysis by HPLC.

Spiking of liver samples

Chicken liver (approximately 10 g) was homogenized without the addition of solvent to ensure homogeneity of endogenous porphyrins throughout sub-samples. Aliquots (100 mg) of the liver homogenate were spiked with 1 *M* HCl solutions (20 μl) of uro-, heptacarboxyl-, hexacarboxyl-, pentacarboxyl- and coproporphyrin at concentrations ranging from approximately 50 to 50 000 pmol/g. Porphyrins were extracted with acetonitrile–HCl, concentrated on Sep-Paks, brought to dryness under nitrogen, reconstituted in HCl and analyzed by HPLC as described above.

Preparation of standard solutions of porphyrins

A few grains of each porphyrin were dissolved in concentrated HCl (1 ml) and diluted to 1 *M* HCl with water (11 ml). Concentrations of uroporphyrin III and coproporphyrin III were determined using millimolar extinction coefficients of 505 (406 nm) and 470 (401 nm), respectively [9]. Millimolar extinction coefficients of 496 (404 nm), 486 (403 nm) and 478 (402 nm) were used to determine concentrations of the type I isomers of heptacarboxyl-, hexacarboxyl-, and pentacarboxylporphyrin standards, respectively [10]. Mixed standards of porphyrins were prepared from 1 *M* HCl solutions of individual porphyrins.

Chromatographic conditions

HPLC was carried out as previously described [7]. In brief, gradient elution was carried out with a sodium phosphate (0.1 *M*, pH 3.5)–methanol mobile phase. The flow-rate was 2.0 ml/min, and injections were made when the mobile phase was methanol–sodium phosphate (45:55, v/v). A linear gradient that was commenced upon injection was held for 2.0 min, and a reverse gradient returned the mobile phase to the starting composition in 1.0 min.

RESULTS AND DISCUSSION

Porphyrin recoveries

For recovery studies, chicken liver was spiked with varying concentrations of porphyrins. Porphyrins were extracted into acetonitrile–HCl, extracts were concentrated to dryness, reconstituted in HCl and analyzed by HPLC as described under Experimental. Typical results are shown in Table I. For this study, comparisons were made between one or two extractions into acetonitrile–HCl. One extraction of uroporphyrin spiked at 50 and 100 pmol/g yielded approximately 100% recovery, but one extraction of uroporphyrin spiked at concentrations that ranged from 500 to 50 000 pmol/g yielded recoveries of 76 to 86%. Two extractions of uroporphyrin spiked at concentrations ranging from 500 to 50 000 pmol/g yielded higher recoveries of 93 to 100%. Similarly, two extractions of heptacarboxyl-, hexacarboxyl-, pentacarboxyl-, and coproporphyrin generally yielded higher recoveries than did one extraction (Table I). Therefore, two extractions were used for further studies. Protoporphyrin recoveries were highly variable (50–90%), and the method can be considered only "semi-quantitative" for this porphyrin.

The acetonitrile eluent from Sep-Paks must be brought to complete dryness to obtain high recoveries ($\geq$90%). Moist samples stored in the freezer yielded recoveries that ranged from approximately 65 to 80% (results not shown).

Stability of extracted porphyrins

Uro-, heptacarboxyl-, hexacarboxyl-, pentacarboxyl-, and coproporphyrin standards that were added to chicken liver, extracted with acetonitrile–HCl, concentrated on Sep-Paks, and brought to dryness as described under Experimental were stable for three weeks (results not shown). Porphyrins reconstituted in HCl were also very stable. Typical results are shown in Fig. 1 (top panel). For this study, uroporphyrin was added to chicken liver at a concentration of approximately 260 pmol/g, extracted with acetonitrile–HCl, concentrated with a Sep-Pak and brought to dryness under nitrogen. The lyophil-

TABLE I

RECOVERIES OF URO-, HEPTACARBOXYL-, HEXACARBOXYL-, PENTACARBOXYL-, AND COPROPORPHYRIN

The porphyrins were added to homogenized chicken liver, extracted with acetonitrile–HCl, concentrated to dryness and analyzed by HPLC as described under Experimental. The data summarize the results of a study that involved duplicate spikes of each concentration of each porphyrin and single HPLC injections of each extract. The concentration of endogenous uroporphyrin (12 pmol/g) was subtracted prior to calculating recoveries. Endogenous heptacarboxyl-, hexacarboxyl-, and pentacarboxylporphyrin were below detection limits.

Amount added (pmol/g)	Recovery (%)	
	One extraction	Two extractions
Uroporphyrin		
50	104	95, 103
100	90, 108	93, 101
500	85, 88	100, 96
5000	82, 88	93
10 000	87, 87	91, 100
50 000	69, 83	98, 103
Heptacarboxylporphyrin		
45	71, 79	97, 110
100	62, 70	86, 93
450	78, 86	97, 100
4500	82, 92	90
10 000	60, 62	89, 96
45 000	70, 84	94, 90
Hexacarboxylporphyrin		
60	73, 84	93, 111
100	84, 88	107, 117
600	78, 85	96
6000	78, 88	97, 113
10 000	78, 78	95, 97
60 000	75, 82	96 (2)
Pentacarboxylporphyrin		
50	71, 87	98, 114
100	72, 76	97, 101
500	81, 87	99, 110
5000	74, 88	99
10 000	67, 69	93, 101
50 000	74, 88	99, 102
Coproporphyrin		
60	61, 68	85, 104
100	68, 72	83, 83
600	83, 85	86, 89
6000	82, 92	93
10 000	67	94, 94
60 000	74, 83	94, 98

ized sample was reconstituted in HCl and analyzed by HPLC. Under these conditions, uroporphyrin appeared stable for at least 22 h when stored in the dark at room temperature (Fig. 1, top panel). HPLC analysis also indicated that heptacarboxyl-, hexacarboxyl-, pentacarboxyl-, and coproporphyrin standards that were spiked, extracted, concentrated, and analyzed in a similar manner were also stable for at least 20 h (results not shown).

The acetonitrile–HCl extraction method was compared with the previously described perchloric acid–methanol method [7]. Uroporphyrin was unstable in perchloric acid–methanol extracts that were eluted from Sep-Paks with methanol (Fig. 1, lower panel). Injection onto the HPLC column immediately after extraction (0 h) revealed a large uroporphyrin peak and a small peak which eluted immediately after uroporphyrin (denoted with an asterisk). No obvious decrease in uroporphyrin was observed within 1 h after extraction (chromatogram not shown), but after 3 h, the uroporphyrin peak was decreased by approximately one third, and the peak denoted with the asterisk was increased over its initial level (Fig. 2, lower panel). After 5 h, uroporphyrin had decreased by approximately two thirds, and two peaks eluted after uroporphyrin. Similar results were observed with heptacarboxyl- and hexacarboxylporphyrin (results not shown). These results confirmed our previously unpublished observations that the perchloric acid–methanol extraction procedure is not suitable for preparing extracts several hours prior to HPLC analysis. Studies to identify the fluorescent compounds produced from the parent porphyrins were not carried out.

Sensitivity and reliability

Current research in our laboratory on the porphyrinogenic effects of halogenated aromatic hydrocarbons and other xenobiotics requires a method that allows measurement of basal concentrations of uroporphyrin in small amounts (< 100 mg) of liver tissues from several species of animals. The chromatograms shown in Fig. 2 indicate that the method described in this paper

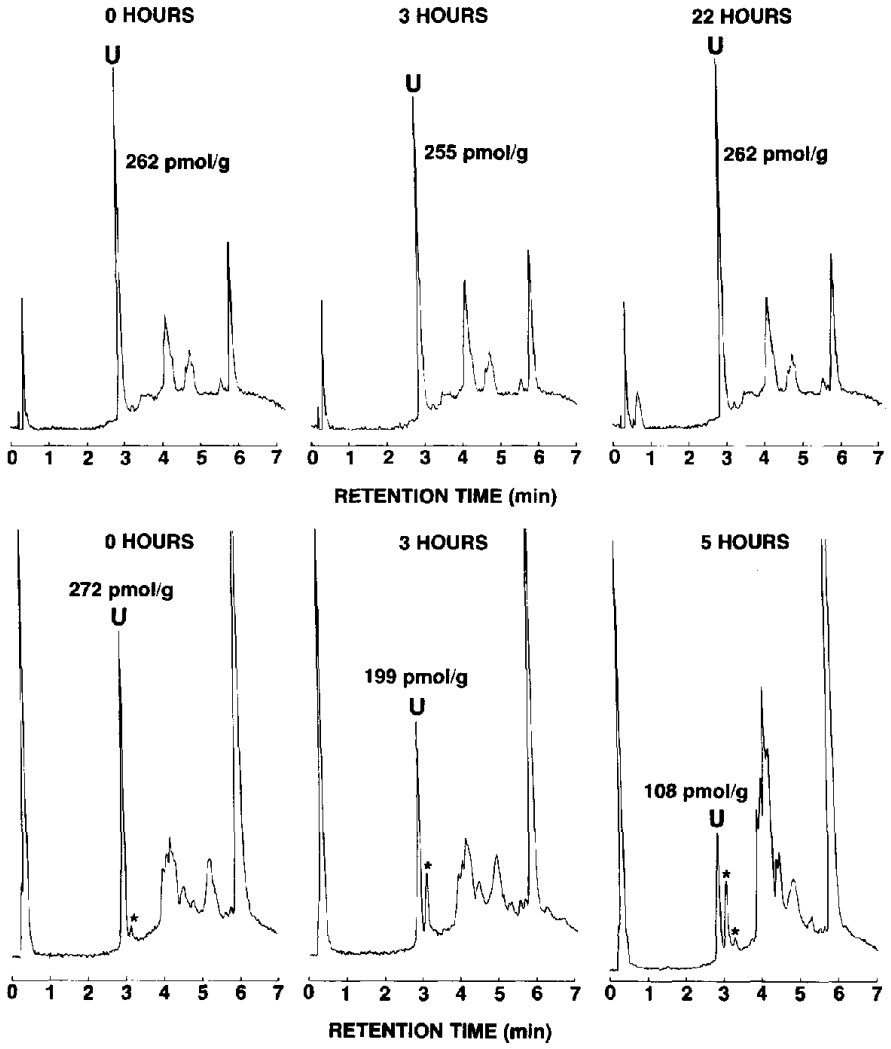

Fig. 1. Chromatograms which compare the stability of uroporphyrin (U) added to chicken liver and extracted using either the acetonitrile–HCl method (top panel) or the perchloric acid–methanol method (bottom panel). Asterisk marks denote unidentified decomposition products of uroporphyrin. Injection volume was 25 μl.

allows quantification of uroporphyrin in 10-mg samples of chicken and rat liver.

Porphyrins were separated and quantified using short and relatively inexpensive HPLC columns that allow rapid separations (injections can be made every 10 min). At least 100 injections of extracts from liver samples weighing from 10 to 100 mg could be carried out without noticeable effects on retention times and peak shapes. Injection volumes were usually 25 μl, but volumes as large as 100 μl could also be injected without noticeable effects on peak shapes. Related studies in our laboratory have shown that the method can also be used to quantify porphyrins in hepatocyte cultures [11] and that it can be used with an HPLC system equipped with an autosampler.

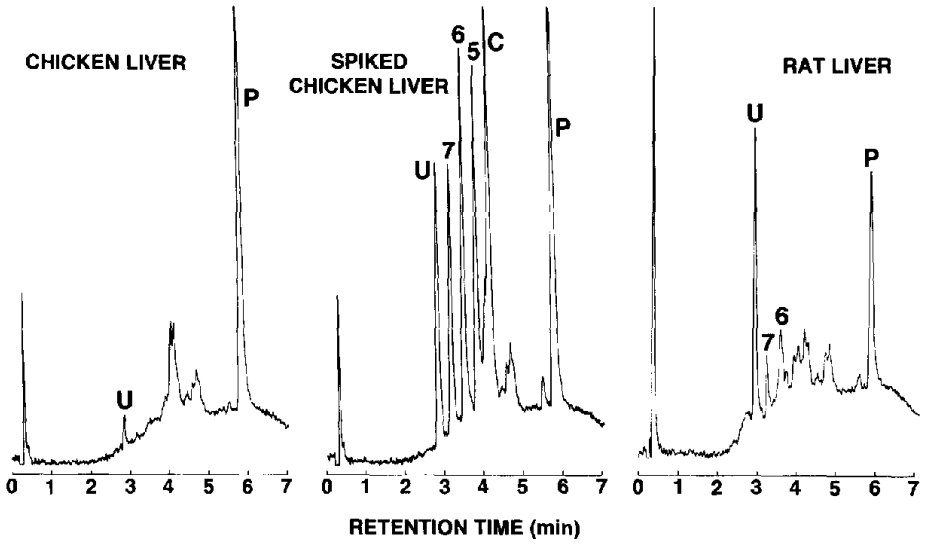

Fig. 2. Chromatograms which show porphyrin patterns in 10-mg samples of (left) unspiked chicken liver, (middle) chicken liver spiked with approximately 100 pmol/g uro- (U), heptacarboxyl- (7), hexacarboxyl- (6), pentacarboxyl- (5), and coproporphyrin (C), and (right) Sprague–Dawley rat liver. The concentration of uroporphyrin in unspiked chicken liver was 12 pmol/g. The concentrations of uro-, heptacarboxyl-, and hexacarboxylporphyrin in rat liver were 113, 18 and 19 pmol/g, respectively. Injection volume was 25 μl.

CONCLUSION

The method described to extract and concentrate porphyrins from liver tissue is a substantial improvement over the perchloric acid–methanol method and over other methods of which we are aware. It allows the extraction of uro-, heptacarboxyl-, hexacarboxyl-, pentacarboxyl-, and coproporphyrin from milligram quantities of tissue with yields of approximately 90–100%. Porphyrins were stable for at least three weeks when dried extracts were stored at −80°C. Therefore, many samples can be extracted prior to analysis by HPLC. Porphyrins reconstituted in HCl were stable at room temperature for at least 20 h.

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