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Short Communication

Determination of individual porphyrins in rodent urine using high-performance liquid chromatography following clean-up by anion-exchange chromatography

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ABSTRACT

We describe a method for the rapid clean-up of rodent urine samples prior to the analysis of porphyrin carboxylic acids by reversed-phase high-performance liquid chromatography (HPLC) with fluorescence detection. A simple pretreatment step using chromatography on a Dowex 1X8 anion-exchange resin effectively removes fluorescent substances that are present in rodent urine and would otherwise interfere with the detection and quantitation of urinary prophyrins by HPLC. Recovery of porphyrins with four to eight carboxyl groups (coproporphyrin to uroporphyrin) averaged 93% using this procedure. The use of this method to determine the amount of individual porphyrins present in the urine of hexachlorobenzene-treated mice is illustrated.

INTRODUCTION

The porphyrias comprise a group of inherited or acquired disorders of the heme biosynthetic pathway [1]. The human disease porphyria cutanea tarda and the porphyria produced by certain halogenated aromatic hydrocarbons are characterized by overproduction and increased excretion of porphyrins containing from two to eight carboxylic acid side-chains [2–4]. The measurement of these porphyrins in biological materials (urine, feces, tissue) constitutes an important diagnostic procedure in the study of these diseases.

A number of methods for the determination of porphyrin levels in biological samples have been described (reviewed in refs. 5 and 6). The most powerful of these involve the use of reversed-phase high-performance liquid chromatography

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(HPLC) with fluorescence detection for separation and identification of the individual porphyrin carboxylic acids [7–17]. For the analysis of human urine samples, acidified aliquots have generally been injected directly onto the HPLC column, with no preparation other than filtration to remove particulate matter [7–13]. In the course of our studies on halogenated aromatic hydrocarbon-induced porphyria in experimental animals [18,19], we found that murine urine contains fluorescent substances that co-elute with the higher carboxylated porphyrins, interfering with their detection and accurate quantitation by HPLC. We therefore adapted an anion-exchange chromatographic procedure for the removal of these interfering substances from rodent urine samples prior to analysis by HPLC.

EXPERIMENTAL

Chemicals

Porphyrin acid chromatographic marker kits were obtained from Porphyrin Products (Logan, UT, U.S.A.). HPLC-grade methanol was from J.T. Baker (Phillipsburg, NJ, U.S.A.). Dowex 1X8-100 (chloride form) was from Sigma (St. Louis, MO, U.S.A.). Other chemicals were as described previously [18].

Animals, treatment and urine collection

Female C57BL/6J mice were purchased from Jackson Labs. (Bar Harbor, ME, U.S.A.). Hepatic porphyria was produced following treatment with iron and hexachlorobenzene as described previously [18]. Urine samples (24 h) were collected from groups of three to five mice, in plastic metabolism cages. Urine samples were preserved with EDTA (*ca.* 4 mg/ml of urine) and sodium carbonate (*ca.* 6 mg/ml) [9], which were present during the collection period.

Sample clean-up

The sample clean-up procedure is adapted from the method described by Martinez and Mills [20]. Urine samples (6–12 ml) were applied to a 7 cm × 0.7 cm I.D. column of Dowex 1X8-100 (maintained at 4°C) that had been previously washed with 3 *M* HCl (3 ml) and equilibrated with deionized water containing NaN_3 (0.1 g/l). After the sample had flowed into the resin, the column was washed with 20 ml of cold 0.1 *M* sodium citrate buffer (pH 4.0 at 4°C). The porphyrins were eluted with two 5-ml aliquots of cold 3 *M* HCl. The column was prepared for the next sample by washing with deionized water (100 ml). We generally used the same column four to eight times (a total of approximately 25–50 ml of urine) before repacking it with fresh Dowex resin.

Separation and quantitation of porphyrins

A 20-μl aliquot of each HCl eluate fraction was analyzed by HPLC [9] using a Varian 5000 liquid chromatograph (Varian Instruments, Palo Alto, CA, U.S.A.)

equipped with a Varian Micropak SP-C_{18}-5 column (150 mm × 4.6 mm I.D.) and a Perkin-Elmer LC-10 fluorescence detector (Perkin-Elmer, Norwalk, CT, U.S.A.) with excitation and emission filters of 360 nm and 500–700 nm, respectively. In addition, an Uptight precolumn 0.5-μm filter (Upchurch Scientific, Oak Harbor, WA, U.S.A.) and a guard column (70 mm × 2.1 mm I.D., packed with Co:Pell ODS; Whatman Chemical Separations, Clifton, NJ, U.S.A.) were placed in front of the analytical column. The columns were maintained at 20°C by a water jacket and circulating water bath (Lauda K-2R refrigerated circulator; Brinkman Instruments, Westbury, NY, U.S.A.). Peaks areas were measured using a Perkin-Elmer LCI-100 laboratory computing integrator. HPLC solvents were prepared using water purified with a Milli-Q system (Millipore, Bedford, MA, U.S.A.). Solvent A consisted of a 50% (v/v) mixture of 0.1 *M* sodium phosphate, pH 3.5–methanol; solvent B was 100% methanol. Both solvents were degassed with helium immediately prior to use. The porphyrins were separated using a 9-min linear gradient from 10 to 100% solvent B (55 to 100% methanol) followed by 6 min at 100% B, at a flow-rate of 1.0 ml/min. The column was re-equilibrated with a 5-min reverse gradient and 10 min at initial conditions. The amount of each porphyrin was determined from a standard curve generated with porphyrin I standards (Porphyrin Products). Peak areas were linearly related to amount injected onto the column over a range of 0.5–50 pmol for each porphyrin.

Recovery experiment

Urine was collected from five untreated C57BL/6J mice as described above. The sample was split into two equal aliquots. A mixture of porphyrin standards (approximately 2 nmol each) in 0.25 *M* HCl was added to one aliquot, and an equal volume of 0.25 *M* HCl was added to the other. Each aliquot was then applied to the Dowex column and the porphyrins were eluted and analyzed as described above. Recovery of added porphyrins was evaluated by comparing the amounts measured in the spiked sample to the sum of the endogenous and added porphyrins.

RESULTS AND DISCUSSION

The original HPLC method of Ford *et al.* [9], applied to samples of human urine, did not include a clean-up step. In agreement with their results and the results of others [8–13], we found that human urine is relatively free of fluorescent contaminants that might interfere with porphyrin determinations; such samples can be analyzed directly by HPLC. In contrast, rodent urine contains several other fluorescent compounds, with elution times similar to those of some porphyrins (Fig. 1A). These compounds interfere with the quantitation of the porphyrins, especially uroporphyrin, by distorting peak shapes and the fluorescence baseline. Several investigators have used ion-exchange chromatography to partially purify porphyrins prior to determination of *total* porphyrin concentrations

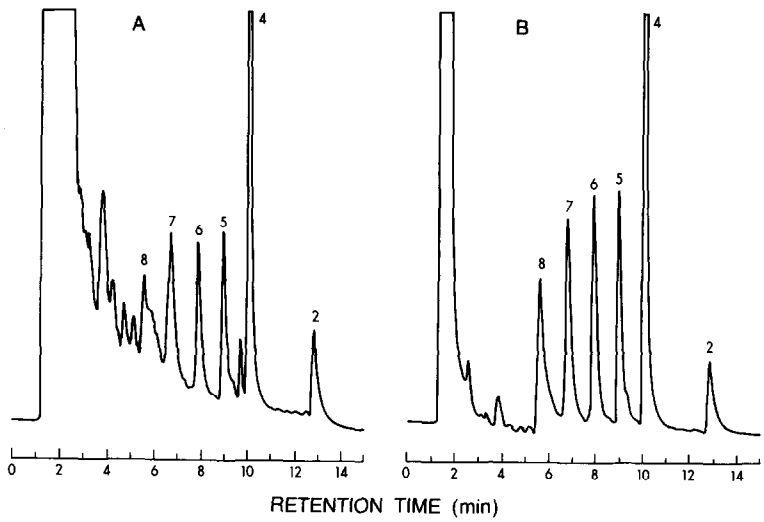

Fig. 1. Urine sample clean-up by ion-exchange chromatography. A mixture of porphyrin standards was added to a urine sample collected from mice, as described in Experimental. (A) An aliquot of the spiked urine was acidified and 20 μl were analyzed by HPLC. (B) An aliquot of the same spiked urine was applied to a column of Dowex 1X8; the column was washed and the porphyrins eluted as described in Experimental. A 20-μl aliquot of the HCl eluate [containing approximately 20 pmol of each porphyrin, except coproporphyrin (35 pmol)] was analyzed by HPLC. The ordinate represents relative fluorescence. Peaks: 8 = uroporphyrin; 7 = heptacarboxylporphyrin; 6 = hexacarboxylporphyrin; 5 = pentacarboxylporphyrin; 4 = coproporphyrin; 2 = mesoporphyrin.

by fluorimetry. We adapted one of these procedures [20] for the removal of interfering compounds prior to the analysis of individual porphyrins by HPLC.

The effectiveness of the clean-up procedure is illustrated in Fig. 1B. Chromatography of mouse urine samples on Dowex 1X8 prior to HPLC provided a flatter baseline and removed the interfering substances. Using this method, the recovery of porphyrins added to a control urine sample varied from 86 to 99%, with a mean of 93% for the five porphyrins (Table I). Fig. 2 illustrates the use of this method for the determination of porphyrins in the urine of mice made porphyric by treatment with hexachlorobenzene [18].

Of several wash buffers (pH 2.0–7.0) evaluated, 0.1 *M* sodium citrate (pH 4.0) was the most effective at removing interfering compounds without affecting the retention of the porphyrins on the anion-exchange resin. Most (85–95%) of the porphyrins were eluted with the first aliquot of 3 *M* HCl. Depending on the need for speed, sensitivity and recovery, the two HCl eluates can be analyzed separately or combined and analyzed together. The HCl eluate(s) can be analyzed directly by HPLC without further treatment. We have found no degradation of our analytical column following more than 250 injections (20 μl each) of the 3 *M* HCl eluates. We routinely replace the precolumn filter every 15–25 runs and repack the guard column after approximately 75 runs.

TABLE I

RECOVERY OF PORPHYRINS FROM MOUSE URINE

A sample of mouse urine was spiked with a mixture of porphyrin standards (*ca.* 10 nmol total porphyrins[a]), cleaned up by anion-exchange chromatography and analyzed by HPLC as described in Experimental. Levels of endogenous porphyrins were determined with an identical sample of unspiked urine. This experiment was repeated once with similar results.

Porphyrin	Amount (nmol)				Recovery (%)
	Endogenous	Added	Expected	Measured	
Uroporphyrin	–[b]	2.15	2.15	1.85	85.9
Heptacarboxylporphyrin	–	1.93	1.93	1.70	88.1
Hexacarboxylporphyrin	–	2.01	2.01	2.00	99.4
Pentacarboxylporphyrin	–	2.27	2.27	2.08	91.8
Coproporphyrin	1.49	1.82	3.31	3.20	96.6
Total	1.49	10.17	11.66	10.81	92.8

[a] This amount of total porphyrins is equivalent to the amount excreted in 24 h by a moderately porphyric C57BL/6J mouse [18].

[b] Below the limit of quantitation (approximately 0.1 nmol).

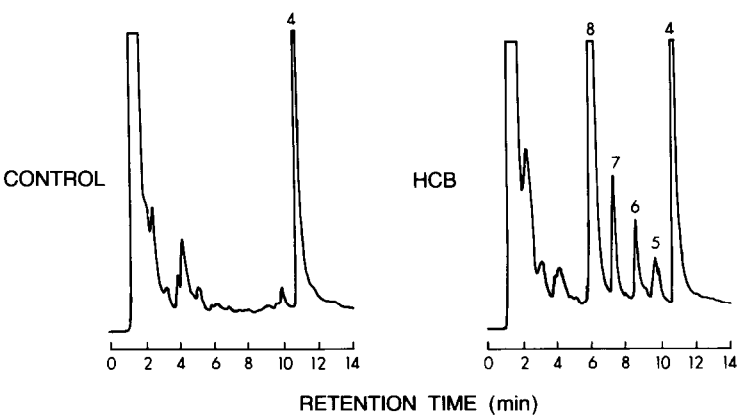

Fig. 2. HPLC profiles of urinary porphyrins from control and hexachlorobenzene-treated mice. Mice were fed control or hexachlorobenzene-containing chow for eight weeks as described previously [18]. Urine samples (pooled 24-h collections from three to five mice per cage) were cleaned up by anion-exchange chromatography and separated by HPLC with fluorescence detection as described in Experimental. Peaks as in Fig. 1. Abbreviation: HCB = hexachlorobenzene.

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